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Report Number AN-1-1983

ION CHANNELS FROM MAMMALIAN BRAIN AND HEART, INCORPORATED INTO PLANAR LIPID BILAYERS: REGULATION BY MEMBRANE POTENTIAL, CALCIUM, AND NEUROTOXINS.

Annual Summary Report

Bruce K. Krueger, Ph.D.

Robert J. French, Ph.D.

September, 1983

Supported by:

U. S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701-5012

Contract No.: DAMD17-82-C-2188

University of Maryland School of Medicine, Contractor
660 West Redwood Street, Baltimore, Maryland 21201

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE	READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. AH50029 — 3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle): Ion channels from mammalian brain and heart, incorporated into planar lipid bilayers: Regulation by membrane potential, calcium, and neurotoxins.	5. TYPE OF REPORT & PERIOD COVERED: Annual Progress Report 8/1/82 - 7/31/83
	6. PERFORMING ORG. REPORT NUMBER: AN-1-1983
7. AUTHOR(s): Bruce K. Krueger; Robert J. French	8. CONTRACT OR GRANT NUMBER(s): DAMD17-82-C-2188
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Departments of Physiology and Biophysics, University of Maryland School of Medicine, 660 W. Redwood St., Baltimore, MD 21201	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: 61102A.3 M161102BS10.AN101
11. CONTROLLING OFFICE NAME AND ADDRESS: U. S. Army Medical Research and Development Command, Fort Detrick, Frederick, MD 21701-5012	12. REPORT DATE: September, 1983
	13. NUMBER OF PAGES: 34
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report): Unclassified.
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

None

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Membranes, ion channels, single channels, sodium, calcium, electrophysiology saxitoxin, tetrodotoxin, batrachotoxin, neurotoxins, Gonyalux.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Sodium channels and calcium channels from rat brain membranes, have been incorporated into planar phospholipid bilayer membranes and characterized electrophysiologically. Currents through many channels (macroscopic currents) and those through single channel molecules (single channel currents) were studied. The sodium channels were activated by the neurotoxin batrachotoxin, and were selective for sodium over potassium, cesium, and chloride. They opened as the membrane was depolarized, and were blocked by nanomolar concentrations of the neurotoxins saxitoxin (STX) and tetrodotoxin (TTX).

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20. Abstract (cont.)

The single channel conductance was 30 pS in symmetrical 0.5 M NaCl, 0.1 mM $CaCl_2$. Block of single sodium channels by STX was found to be dependent on the membrane potential with depolarizing potentials reducing the potency of STX block by as much as 50-fold. Both blocking and unblocking rate constants were affected by the membrane potential: depolarization decreased the rate (probability) of channel block by STX and increased the rate (probability) of unblock. These sodium channels are responsible for depolarizing phase of the action potential in nerve and muscle cells and appear to constitute the sole site of action of STX and TTX. Single calcium channels from rat brain membrane vesicles were also incorporated into planar bilayers; both macroscopic and single channel calcium currents were studied. The calcium channels were selective for calcium, barium, and strontium over monovalent cations and anions. The single channel conductances for Ca^{++}, Ba^{++}, and Sr^{++} were 5 pS, 8.5 pS and 5 pS, respectively, in symmetrical 0.25 M $Me^{++}Cl_2$. Membrane depolarization increased the probability of channel opening and decreased the probability of channel closing. There was an apparent reciprocal relationship between the single channel conductance and the mean open lifetime for the three permeant cations tested, suggesting a possible relationship between channel gating (voltage dependence) and ion permeation. These calcium channels may be the pathways for calcium entry during stimulus-coupled release of neurotransmitter at synapses in the central nervous system.

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SUMMARY

Sodium channels and calcium channels from rat brain membranes, have been incorporated into planar phospholipid bilayer membranes and characterized electrophysiologically. Currents through many channels (macroscopic currents) and those through single channel molecules (single channel currents) were studied. The sodium channels were activated by the neurotoxin batrachotoxin, and were selective for sodium over potassium, cesium, and chloride. They opened as the membrane was depolarized, and were blocked by nanomolar concentrations of the neurotoxins saxitoxin (STX) and tetrodotoxin (TTX). The single channel conductance was 30 pS in symmetrical 0.5 M NaCl, 0.1 mM $CaCl_2$. Block of single sodium channels by STX was found to be dependent on the membrane potential with depolarizing potentials reducing the potency of STX block by as much as 50-fold. Both blocking and unblocking rate constants were affected by the membrane potential: depolarization decreased the rate (probability) of channel block by STX and increased the rate (probability) of unblock. These sodium channels are responsible for depolarizing phase of the action potential in nerve and muscle cells and appear to constitute the sole site of action of STX and TTX. Single calcium channels from rat brain membrane vesicles were also incorporated into planar bilayers; both macroscopic and single channel calcium currents were studied. The calcium channels were selective for calcium, barium, and strontium over monovalent cations and anions. The single channel conductances for Ca^{++}, Ba^{++}, and Sr^{++} were 5 pS, 8.5 pS, and 5 pS, respectively, in symmetrical 0.25 M $Me^{++}Cl_2$. Membrane depolarization increased the probability of channel opening and decreased the probability of channel closing. There was an apparent reciprocal relationship between the single channel conductance and the mean open lifetime for the three permeant cations tested, suggesting a possible relationship between channel gating (voltage dependence) and ion permeation. These calcium channels may be the pathways for calcium entry during stimulus-coupled release of neurotransmitter at synapses in the central nervous system.

*Originator Furnished Keywords Include:

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

TABLE C ONTENTS

EXPERIMENTAL RESULTS

(for the period 8/1/82 through 7/31/83)

A. SPECIFIC AIMS OF LAST PROPOSAL.

In this section of the report we list the Specific Aims of our original proposal (for three years). The subsequent comments summarize the progress made (if any) on each Specific Aim during the first year of the project.

1. To develop and refine methods to study ion selective channels from excitable membranes incorporated into planar bilayers.

During the first year of the project, we have developed the capability to study two different types of ion channels incorporated into planar bilayers. New electronic circuits, designed and built by our group, provide higher resolution and frequency response as well as capacity compensation. Further improvements are in progress. We now have four set-ups in operation, two of which are interfaced to microcomputers. Software has been developed to analyze single channel fluctuations due to voltage-gating of single channels and to the blocking and unblocking of the channels by STX. Both "painted" and solvent-free "folded" planar bilayers are in use.

2. To survey the diversity of other ion channels incorporated into bilayers from neuronal and cardiac muscle preparations, including other voltage-gated channels, calcium-activated channels, and channels activated by putative neurotransmitter substances.

We have identified and characterized voltage-dependent calcium channels in planar bilayers exposed to rat brain membrane vesicles. The results are summarized below (see Nelson et al., 1984, ref. 29)

3. To study voltage-dependent, saxitoxon (STX)-sensitive sodium channels from rat brain membrane incorporated into lipid bilayers.

As summarized below in Krueger et al. (1983) (7) and French et al. (1984) (15), we have characterized the reconstituted sodium channels with respect to voltage dependence and ion selectivity. Of particular interest was the finding that block of single sodium channels by STX was voltage-dependent with block being up to 50-fold less potent at depolarized potentials.

4. To provide a definitive characterization of the calcium-activated potassium channel from rat brain.

This part of the proposal has been dropped in order to devote full time to the study of sodium and calcium channels.

5. To characterize, in bilayers, channels incorporated from myocardial membranes, focusing in particular on the role that calcium plays as a charge carrier and modulator of conductances in cardiac membranes.

This phase of the project has been deferred to the second year and preliminary experiments are in progress.

B. PUBLICATIONS AND SCIENTIFIC MEETINGS.

Work under this contract has resulted in three publications:

Krueger, B.K., J.F. Worley, III, and R.J. French. Single sodium channels from rat brain incorporated into planar lipid bilayer membranes. Nature 303: 172-175 (1983).

French, R.J., J.F. Worley, III, and B.K. Krueger. Voltage-dependent block by saxitoxin of sodium channels incorporated into planar lipid bilayers. Biophysical Journal 45: 301-310 (1984).

Nelson, M.T., R.J. French, and B.K. Krueger. Single calcium channels from rat brain in planar lipid bilayers. Nature 308: 77-80 (1984).

Each section of the report below, will consist of a brief summary of one of the above components of the project together with conclusions and future plans, followed by a complete text of the resulting publication.

Staff members on this project attended the following scientific meetings to present the experimental results from this project:

R.J. French. Biophysical Society Meeting, San Diego, CA. 2/83.

M.T. Nelson. Biophysical Society Meeting, San Diego, CA. 2/83.

B.K. Krueger. Gordon Research Conference, Molecular Pharmacology, 6/83 (Invited Speaker).

R.J. French. Gordon Research Conference, Cell Membranes, 7/83 (Invited Speaker).

R.J. French. International Physiological Congress, Sydney, Australia, 8-9/83 (Invited Speaker).

The work described in French et al. (1984) was solicited as a Plenary Discussion Paper for the Fourth Biophysical Discussion, Airlie, VA, 10/83. R.J. French, J.F. Worley, M.T. Nelson, and B.K. Krueger were invited to attend the conference.

C. SODIUM CHANNELS IN PLANAR BILAYERS.

Summary. Voltage-dependent sodium channels from rat brain were incorporated into planar phospholipid bilayer membranes. The channels were activated by batrachotoxin (BTX) on the side opposite rat brain membrane addition (trans) and were blocked by saxitoxin (STX) from the side of vesicle addition (cis). This allowed the assignment of the cis side as the outside of the bilayer with respect to the channels. Both macroscopic (multichannel) and single channel currents were studied. The single channel conductance was 30 pS (30×10^{-12} $ohms^{-1}$) in symmetrical 0.5 M NaCl. STX blocked with an apparent dissociation constant of about 4 nM. Stepwise, unitary current fluctuations were observed which were due to the blocking and unblocking of individual sodium channels. Single BTX-activated sodium channels were selective for sodium over potassium, cesium, and chloride. Hyperpolarization favored channel closing. Block of single sodium channels by STX was voltage-dependent with hyperpolarizing potentials favoring block.

The results described above followed from preliminary results obtained with membrane vesicles from rat brain, in which calcium-activated potassium channels were observed in planar phospholipid bilayers exposed to the brain vesicles (Krueger, French, Blaustein, and Worley, abstract in Biophys. J. 37: 170a, 1982). We knew that the vesicles also contained sodium channels because binding of ^{3}H-saxitoxin (STX) was enriched in the vesicles (1). When the protocol of Miller (2) was used, with the exceptions that NaCl replaced KCl and brain vesicles were added, a STX-blockable conductance appeared in the bilayers. This result also required that batrachotoxin (BTX) be present on the side opposite vesicle addition. BTX inhibits sodium channels inactivation and causes the channels to remain open at the normal resting potential (3-6). Further study indicated that under appropriate conditions (low vesicle concentration) a single channel could be incorporated into the bilayer and studied for some time. We characterized these BTX-activated sodium channels at the single channel level with respect to potency of STX block, ion selectivity, and voltage dependence. Details of these results can be found in the publication that follows (7). There seems to be little doubt that these channels are identical to those that are responsible for the depolarizing phase of the action potential of nerve cells and that are the only know site of action of STX and TTX.

Several particularly interesting findings came out of this work. This was the first report in the literature of the successful incorporation of voltage-dependent sodium channels into an artificial membrane. There had been some concern that the decane present in the planar bilayers (8) might alter the function of the channels, however, the properties of the channels are remarkably similar to those of normal and BTX-activated sodium channels in intact cells (4,6,9-12) including single channel parameters (10-12) studied by the patch clamp technique (13). Recently, this conclusion was supported by the demonstration that nonane and decane do not affect sodium channels in squid axons (14). By taking advantage of the fact that in the presence of BTX, the channels are nearly always open at membrane potentials

of ±60 mV, we demonstrated stepwise, unitary current fluctuations due to the blocking and unblocking of individual sodium channels by STX as shown in figure 2 of ref. 7. This demonstrated for the first time that block by STX is all-or-none, that is, a channel is either fully open or completely blocked by STX. Another interesting result that is described in more detail in the next section and (15) is that block by STX is affected by the membrane potential with depolarizing potentials reducing the potency of block by up to 50-fold.

The results obtained are particularly encouraging, not only because they provide additional detailed information about the functional properties of sodium channels and about STX block, but also because they suggest a method for assaying the function of channels that have been removed from nerve membranes by detergent treatment and subjected to biochemical manipulations and purification. Several groups have purified the STX binding site from mammalian nerve and muscle membranes (16,17) and have also reconstituted the purified binding sites in phospholipid vesicles by removing the detergent. These reconstituted binding sites exhibited BTX activated ^{22}Na fluxes that were blocked by STX (18,19). This method lacks control over the membrane potential, and even using a rapid mixing device (20), time resolution is limited to about 50-100 msec. Reconstitution of sodium channels in planar bilayers as we have described, provides the means to assay function on a physiological time scale with control over the membrane potential. Research is under way in Dr. Krueger's lab, with support from NIH, to purify STX binding sites and to assay function of these purified channels by reconstitution in planar bilayers.

Technical note: The traditional voltage convention in planar bilayer publications was used in ref. 7 (i.e., the reference potential was that of the trans side). It was found that all of the channels incorporated into the bilayer with their STX blocking sites facing the cis side, allowing us to define cis as the extracellular side with respect to the channels. Membrane potentials reported in ref. 7 are therefore opposite to the normal cellular convention (outside at reference potential). Data reported in ref. 15 are given in the normal cellular convention.

Reprinted from Nature, Vol. 303, No. 5913, pp. 172-175, 12 May 1983

Single sodium channels from rat brain incorporated into planar lipid bilayer membranes

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A voltage- and time-dependent conductance for sodium ions is responsible for the generation of impulses in most nerve and muscle cells[1]. Changes in the sodium conductance are produced by the opening and closing of many discrete transmembrane channels[2]. We present here the first report of electrical recordings from voltage-dependent sodium channels incorporated into planar lipid bilayers. In bilayers with many channels, batrachotoxin[3] (BTX) induced a steady-state sodium current that was blocked by saxitoxin[4] (STX) at nanomolar concentrations. All channels appeared in the bilayer with their STX blocking sites facing the side of vesicle addition, allowing us to define that as the extracellular side. Current fluctuations due to the opening and closing of single BTX-activated sodium channels were voltage-dependent (unit conductance, 30 pS in 0.5 M NaCl); the channels closed at large hyperpolarizing potentials. Slower fluctuations of the same amplitude, due to the blocking and unblocking of individual channels, were seen after addition of STX. Block of the sodium channels by STX was voltage-dependent, with hyperpolarizing potentials favouring block. The voltage-dependence gating, ionic selectivity and neurotoxin sensitivity suggest that these are the channels that normally underlie the sodium conductance change during the nerve impulse.

Planar bilayers were formed by the method of Mueller *et al.*[5] across a 0.25–0.5 mm diameter hole in a polystyrene partition separating two chambers, both normally containing 0.5 M NaCl, 10 mM HEPES, 0.15 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.05 mM EGTA, pH 7. The membrane-forming solution contained 33 mg ml^{-1} phosphatidylethanolamine (bovine brain; Avanti Polar Lipids) and 13 mg ml^{-1} phosphatidylserine (bovine brain; Avanti) in decane. The current across the bilayer was measured and command voltages were applied to the bathing solutions via a pair of Ag/AgCl electrodes. The side to which vesicles were added was designated the *cis* side; the opposite (*trans*) side was held at virtual ground. The area-specific resistance of the bilayer before addition of biological material was $\sim 10^6\ \Omega\ cm^2$; the cutoff frequency of the current-to-voltage converter was ~80 Hz. All experiments were performed at ambient temperature (22–25 °C).

Membrane vesicles were prepared from rat brain homogenates as described by Krueger *et al.*[6]. Briefly, fresh rat forebrains were homogenized in isotonic sucrose, using a cavitating tissue disrupter. Low-speed (1,000*g*) and intermediate-speed (10,000*g*) pellets were discarded and the high-speed (100,000*g*) pellet was suspended in 0.4 M sucrose and stored at −77 °C for up to 6 months before use. The specific activity of ^{3}H-STX binding was enriched about fivefold in this fraction compared with the crude homogenate; at least 60% of the binding sites were recovered in this fraction. We have successfully incorporated sodium channels from at least 12 different membrane preparations obtained by this method.

Incorporation of sodium channels was achieved by the 'fusion' method[7–9]. Addition of membrane vesicles to the *cis* chamber, with 0.6 μM BTX present on the *trans* side, resulted in stepwise increases in conductance. After 5–30 min, the conductance of the membrane increased by 30–1,000 pS. Before addition of membrane vesicles, the conductance was 10–20 pS. Both BTX and vesicles were required to produce the conductance increase. Figure 1*a* shows the current across a planar bilayer following addition of membrane vesicles (10 μg ml^{-1}) as described above. About 15 pA flowed across the membrane at ±30 mV; a steady-state, voltage-independent sodium conductance is not surprising because, in the presence of BTX, sodium channels in nerve close only when the membrane is hyperpolarized beyond −90 mV (refs 10, 11 and see below). Addition of 0.3 μM STX to the *cis* side resulted in almost a complete block of the membrane conductance. In this experiment, the conductance in the presence of STX was nearly identical to that seen before incorporation of membrane vesicles. In general, the number of channels incorporated and the rate of incorporation are dependent on the concentration of membrane protein added to the *cis* side. At high concentrations (10 μg ml^{-1}) several channels usually appear in the bilayer within 5 min. In order to obtain only a single channel or very few channels, very low protein concentrations (0.2 μg ml^{-1}) are used and longer periods (up to 30 min) are required. Channels can usually be studied for at least 30 min after incorporation; in some cases a single membrane with one or a few channels has been studied for 2 h, with repeated changes in ionic conditions by perfusion of the *cis* chamber.

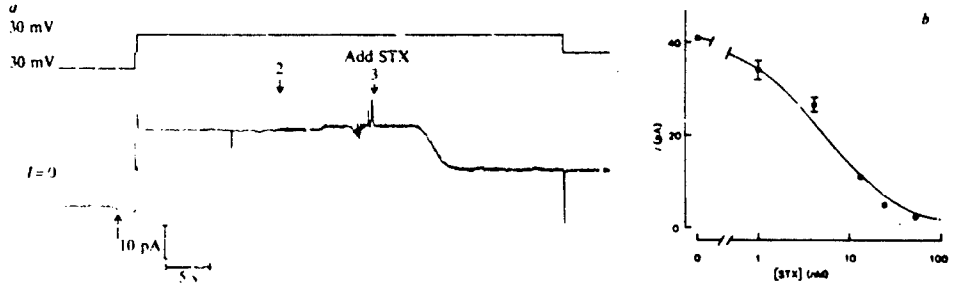

Fig. 1 *a*, Block of conductance by STX. Rat brain membrane vesicles were incorporated into the planar bilayer as described in the text, with 0.5 M NaCl on both sides and 0.6 μM BTX on the *trans* side. As the ion concentrations on both sides of the membrane were identical, the reversal (zero-current) potential was 0 mV. Before the beginning of this record, the conductance had increased in several discrete jumps (one such jump can be seen at arrow 1). With the membrane potential held at +30 mV, both chambers were stirred (beginning at arrow 2) and 0.3 μM STX was added to the *cis* side (arrow 3). The disturbance just before STX addition was due to insertion of the pipette into the *cis* chamber. The current record was filtered (low-pass) at 2 Hz. *b*, Dose–response curve for STX block. Same conditions as in *a* except that the STX concentration on the *cis* side was raised in increments (E_m = +30 mV). In this case, the data have been corrected by subtracting a 20-pA, STX-independent current (5 μM STX *cis* and *trans*). The curve was drawn for a blocker with a dissociation constant of 5 nM. The bars indicate standard errors.

Figure 1*b* shows the effect of varying concentrations of STX (*cis*) on the conductance of the bilayer. Half-maximal block of the conductance occurred at about 5 nM STX (at +30 mV), which is similar to the K_i for block of macroscopic sodium currents in nerve by STX[12-14] and to the K_d for binding of radiolabelled STX[4,15]. Block of the conductance by STX was completely reversed by perfusing the *cis* chamber with toxin-free solution. Addition of up to 1 μM STX to the *trans* side did not affect the membrane conductance. This sidedness of STX block indicates that the channels were incorporated into the bilayer with their extracellular sides facing *cis*.

Figure 2*a* shows a current record taken after exposure of the bilayer to membrane vesicles in the presence of BTX, and subsequent block by 0.3, 1.3 and 5 μM STX (*cis*). The stepwise fluctuations in the presence of STX seemed to reflect the blocking and unblocking of individual sodium channels, because the mean duration of the conducting state was substantially shortened by raising the STX concentration on the *cis* side. Measurement of the mean times in the zero- and one-channel conductance levels at +30 mV, indicated that the off-rate and on-rate constants for STX block were 0.05 s^{-1} and $1.1 \times 10^7\ s^{-1}\ M^{-1}$, respectively, implying a dissociation constant of 4.5 nM (compare with Fig. 1*b*). In many experiments, with very few channels incorporated, discrete, stepwise conductance fluctuations of similar magnitude were also observed in the absence of STX. Examples of these single-channel fluctuations can be seen in Figs 2*a*, *b* and 3 (insets).

The unexpected observation that block of BTX-activated sodium channels by STX was voltage dependent is shown in Fig. 2*b*. After addition of 0.32 μM STX to the *cis* side of a membrane containing seven sodium channels, the channels remained conducting about 5% of the time at −30 mV but only 1% of the time at +30 mV. There was an even more dramatic difference between the percentage block at −60 mV and that at +60 mV (see Fig. 2 legend). Thus, voltages that would enhance entry of STX (a divalent cation) into the channel favour block. Although block of cardiac sodium channels by TTX has been reported to be voltage dependent[16], this conclusion has been challenged on technical grounds[17] and is not supported by more recent voltage-clamp studies[18,19]. Such an effect has not been reported in neurones. However, because of the long relaxation times for STX block ($\tau = 1$ s at $K_d = 5$ nM; [STX] = 100 nM), the magnitude of the peak sodium current during a single voltage-clamp pulse reflects the degree of block at the preceding holding potential rather than at the pulse potential. Even the complex pulse protocols required to test for steady-state voltage-dependent STX block of sodium channels without BTX treatment[6], can be used to explore only a limited voltage

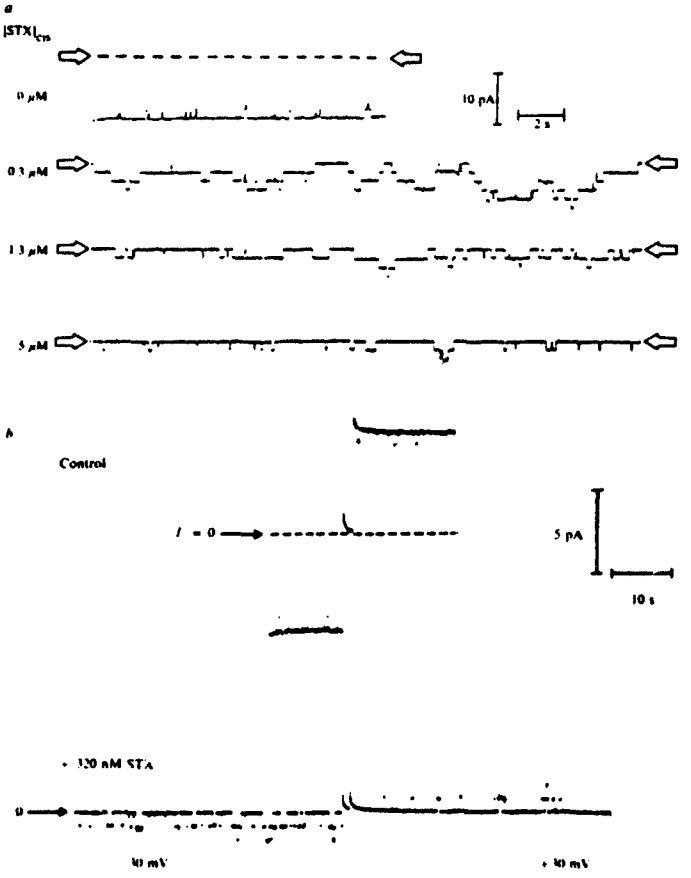

Fig. 2 *a*, Single sodium channel current fluctuations induced by STX. Sodium channels were incorporated into the bilayer as described in Fig. 1 legend. Top: control trace at −60 mV. Lower traces: same membrane at −60 mV following addition of 0.3 μM, 1.3 μM and 5 μM STX to the *cis* side. The arrows indicate the zero-current level for each record. The current records were filtered (low-pass) at 20 Hz on playback. *b*, Voltage dependence of STX block. Same membrane as in *a* above before (top) and after (bottom) addition of 0.32 μM STX to the *cis* side (voltages shown at bottom refer to both traces). BTX (0.6 μM) was present on the *trans* side. The current records were filtered at 20 Hz on playback. The fraction of channels that remained conducting at various potentials is given below:

Potential (*cis*–*trans*)	% Not blocked
+60 mV	0.7
+30 mV	1.2
−30 mV	5.3
−60 mV	22.6

range. Our ability to examine the steady-state voltage dependence of STX block over a wide range of potentials is a consequence of the inhibition of sodium channel inactivation by BTX. We cannot exclude the possibility that the high sodium concentration or channel modification by BTX are necessary for the STX action to be voltage dependent.

Figure 3 illustrates the selectivity of single BTX-activated channels. In this experiment, the *cis* chamber was perfused with a vesicle-free solution containing 0.1 M NaCl, 0.4 M KCl. The *trans* chamber contained 0.5 M NaCl and 0.6 μM BTX. The reversal potential for the single-channel currents in this experiment was about +36 mV (E_{Na} = 41 mV) which implies a permeability ratio P_K/P_{Na} of 0.06 (mean of 0.07 ± 0.02 (s.e.) in three experiments). In a similar experiment using CsCl instead of KCl, we were unable to detect any permeability to Cs (that is, the zero-current potential approximated E_{Na}). The P_K/P_{Na} ratio is lower than that obtained for BTX-modified channels in the node of Ranvier[19] and in neuroblastoma cells in culture[20,21], and is comparable with a P_K/P_{Na} of about 0.08 for unmodified channels in the node of Ranvier[19,22]. In general, permeability ratios depend on the ionic composition of the bathing solutions[23,24], and can even differ significantly between two related cell lines[20].

The single-channel current fluctuations in symmetrical 0.5 M NaCl (Fig. 3, open circles) revealed a unit conductance of about 30 pS. This value is consistent with the reported value of about 15–18 pS using the patch-clamp technique[2,25] (but see ref. 26), considering the much higher sodium concentration used in our experiments and assuming a saturating conductance–activity relationship[27]. The single-channel conductance with 0.1 M NaCl and 0.4 M KCl on the *cis* side and 0.5 M NaCl on the *trans* side (Fig. 3, closed circles) was 23 pS, which was somewhat lower than in symmetrical 0.5 M NaCl solutions.

Figure 4 illustrates the voltage dependence of a single BTX-activated sodium channel incorporated into a planar bilayer. At potentials less than +70 mV, the sodium channel was open >99% of the time; at more positive potentials, the channel began to close for brief intervals, many of which were too short to be fully resolved by our amplifier. At very large potentials

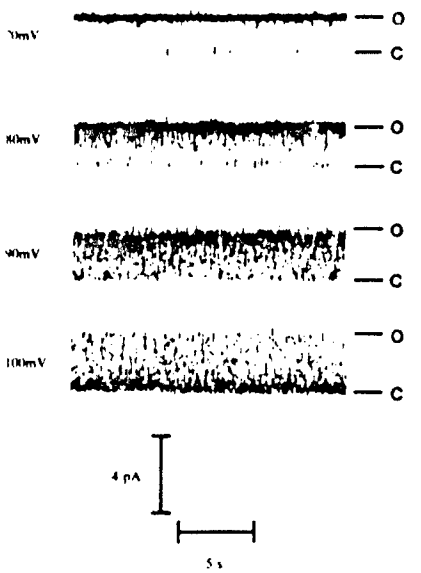

Fig. 4 Voltage dependence of BTX-activated channels. A single sodium channel was incorporated in the planar bilayer as described above. Records are shown at +70, +80, +90 and +100 mV (*cis*–*trans*) as indicated. 'C' and 'O' indicate the closed- and open-state current levels, respectively. The zero-current level for each record was −0.3–0.5 pA below the closed-state level. Records were filtered at 60 Hz on playback. BTX (0.6 μM) was present on the *trans* side. Similar results were obtained in three experiments using single-channel bilayers from two different membrane preparations.

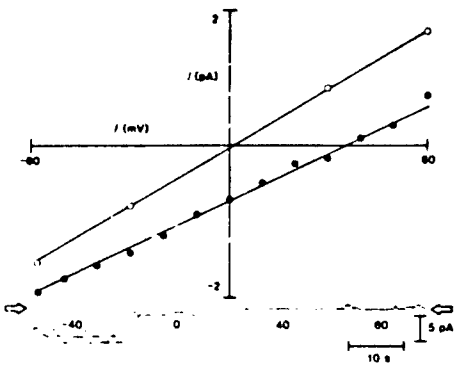

Fig. 3 Current–voltage relationships for single sodium channels. Open circles show the single-channel currents in symmetrical 0.5 M NaCl. Closed circles show the single-channel currents with 0.5 M NaCl (*trans*) and 0.1 M NaCl, 0.4 M KCl (*cis*). BTX (0.6 μM) was present on the *trans* side. The lines are least-squares fits to the data points giving single-channel conductances of 23 pS (closed circles) and 29 pS (open circles). The insets show representative current records taken at −40, 0, +40 and +60 mV for 0.5 M NaCl (*trans*) and 0.1 M NaCl, 0.4 M KCl (*cis*). Current records in insets were filtered (low-pass) at 30 Hz.

the channel was closed almost all the time. The midpoint of the conductance–voltage curve was about +95 mV. As the sodium channels were oriented in the bilayer with their extracellular sides (STX-blocking sites) facing *cis*, positive potentials across the bilayer corresponded to (cell interior) negative potentials *in situ*.

The specific block of the conductance by STX at nanomolar concentrations, the activation of the channels by BTX, the selectivity of the channels for sodium over potassium, and the voltage dependence together with the unit conductance, indicate that these channels are the well studied sodium channels of excitable cells. As the planar bilayer system allows control of the solutions on both sides of the membrane and the lipid composition of the bilayer, it should be useful for biochemically modifying specific sites on the channels as well as for characterizing purified sodium channels.

We thank Marc B. Blaustein and Edouard L. Noisin for excellent technical assistance, Mr G. Ay for making the chambers, Mr J. Michael for assisting with the electronics, Dr J. Daly for a generous supply of BTX, Drs B. E. Alger, M. P. Blaustein, L. Goldman, W. J. Lederer, L. J. Mullins, D. A. Nachshen, and M. T. Nelson for comments on the manuscript. We particularly thank Drs E. Bamberg, R. Coronado, R. Latorre, C. Miller, G. Szabo and R. Waldbilig for introducing us to the mystique of the bilayer. This work was supported by NIH grants NS-16285 (B.K.K) and NS-16775 (R.J.F), by an A. P. Sloan Foundation fellowship (B.K.K.), and by contract DAMD-17-C-2188 from the US Army Research and Development Command (B.K.K. and R.J.F.).

Received 20 January, accepted 8 March 1983

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D. VOLTAGE-DEPENDENT BLOCK OF SODIUM CHANNELS BY SAXITOXIN.

Summary. Our preliminary finding, reported in ref. 7, that the potency of block of sodium channels by STX varied with the membrane potential was investigated in detail in French et al., (1984) (15). All experiments were conducted on bilayers containing only one or a few sodium channels. The voltage dependence of STX block was evaluated from steady state measurements of the fractional block as a function of STX concentration over a range of membrane potentials. It was found that the dissociation constant (K_I) for STX block ranged from about 1 nM at -60 mV (near the normal resting potential) to about 50 nM at +60 mV. By evaluating the kinetic parameters of unitary single channel fluctuations induced by STX, we were able to determine that depolarization decreased the blocking rate constant and increased the unblocking rate constant. At each potential, the K_I derived from kinetic parameters agreed well with the K_I obtained from steady state determinations. We have subsequently found that block of sodium channels by TTX is also voltage-dependent and that the steepness of the K_I vs. potential relationship is about the same as for STX.

Voltage-dependent STX block was entirely unexpected. Several investigators had previously considered the possibility that STX or TTX block was voltage-dependent (7,21-23) but in each case no evidence could be found to demonstrate such an effect of voltage on block. One report (24) suggested that depolarization of heart cells increased the potency of TTX block, however, the validity of the experimental methods used to carry out those experiments have been questioned (25), and more recent studies have failed to confirm that finding (26). As summarized above, our results demonstrate that depolarization reduces the potency of STX block at least under the specific experimental conditions used in our experiments.

A voltage-dependent process suggests that one or more electric charges are interacting with the membrane electric field. The most likely candidate for those charges is the STX molecule itself which is a divalent cation under physiological conditions. One possible mechanism is that the binding site for STX lies inside the channel at a location within the membrane electric field. Thus block by STX, approaching from the outside, would be favored by negative-inside (hyperpolarizing) potentials, making the potency of block greater (27,28). Our results are consistent with this model except that binding of STX to the channels should be voltage-dependent under virtually all conditions; binding of radiolabeled STX and TTX to sodium channels has been shown to be independent of membrane potential (7,21). A second possibility is that STX or TTX in its binding site can be influenced, electrostatically, by another cation (e.g., Na^+ or Ca^{++}) residing at another site within the channel, even if the STX binding site were not inside the membrane field. Occupancy of the second site would disfavor occupancy of the STX binding site by repulsion of like charges. With this model, if the Na/Ca binding site were within the membrane field, the voltage dependence of STX binding would arise indirectly from the voltage dependence of Na/Ca binding. In the renewal proposal for this contract, we proposed a number of experiments that should allow us to determine whether one of these two mechanisms for voltage dependence of STX block is correct. Another question for investigations is whether STX block is voltage dependent only in BTX-activated sodium channels or whether the phenomenon occurs in normal channels as well. Experiments were proposed to answer this question as well.

VOLTAGE-DEPENDENT BLOCK BY SAXITOXIN OF SODIUM CHANNELS INCORPORATED INTO PLANAR LIPID BILAYERS

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ABSTRACT We have previously studied single, voltage-dependent, saxitoxin-(STX) blockable sodium channels from rat brain in planar lipid bilayers, and found that channel block by STX was voltage-dependent. Here we describe the effect of voltage on the degree of block and on the kinetics of the blocking reaction. From their voltage dependence and kinetics, it was possible to distinguish single-channel current fluctuations due to blocking and unblocking of the channels by STX from those caused by intrinsic channel gating. The use of batrachotoxin (BTX) to inhibit sodium-channel inactivation allowed recordings of stationary fluctuations over extended periods of time. In a range of membrane potentials where the channels were open >98% of the time, STX block was voltage-dependent, provided sufficient time was allowed to reach a steady state. Hyperpolarizing potentials favored block. Both association (blocking) and dissociation (unblocking) rate constants were voltage-dependent. The equilibrium dissociation constants computed from the association and dissociation rate constants for STX block were about the same as those determined from the steady-state fractional reduction in current. The steepness of the voltage dependence was consistent with the divalent toxin sensing 30–40% of the transmembrane potential.

INTRODUCTION

Recently, we reported that single sodium channels from rat brain could be incorporated into planar lipid bilayers (Krueger et al., 1983). In the presence of the neurotoxin batrachotoxin (BTX), unitary current fluctuations resulting from the opening and closing of individual channels were observed. The reconstituted sodium channels, closed at hyperpolarizing potentials, were selective for sodium over potassium or cesium, and were blocked by nanomolar concentrations of STX. In that study we found that block of sodium channels by STX was voltage-dependent, with hyperpolarizing potentials favoring block. Here we present a more detailed analysis of this voltage dependence.

An accurate description of the effect of voltage on the toxin-induced block is an essential step toward understanding the molecular details of the toxin's action. The voltage dependence could be due to the passage of the divalent toxin through part of the transmembrane voltage as it approaches or leaves its binding site. Although our data are consistent with this simple picture, the actual physical basis of the voltage dependence could be more complex. For example, an obligatory voltage-dependent reaction, such as a gating transition or the vacating of a nearby ionic binding site, could be associated with toxin binding. Evaluation of the voltage dependence under a variety of conditions will be needed to choose among the different alternatives. These data should permit determination of the position, within the transmembrane field, of the toxin binding site, or identification of the nature of an associated voltage-dependent reaction.

Because sodium channels normally inactivate within a few milliseconds after opening, peak sodium currents during depolarizing steps from hyperpolarized holding potentials are normally used to determine channel availability for conduction. Voltage-dependent block by TTX or STX would be missed if such a voltage-clamp method were used to study the degree of block. Because of the slow association and dissociation rates for STX and TTX binding, a relaxation time of several seconds, at nanomolar concentrations, is expected for a new steady-state block to be reached. Thus, the decrease in peak sodium current during a single test pulse indicates the degree of block at the holding potential, because the block cannot re-equilibrate within the duration of the test pulse. The use of BTX, which inhibits sodium channel inactivation (Albuquerque et al., 1976; Khodorov, 1978; Huang et al., 1982), has allowed us to study the voltage dependence of block by STX in the steady state.

MATERIALS AND METHODS

Planar Bilaye. Formation

Planar bilayers were formed by the technique of Mueller et al. (1963) across a hole (0.25 mm diam) in a polystyrene partition separating solutions containing 0.5 M NaCl, 0.15 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.05

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mM EGTA, 10 mM Na-HEPES, pH 7.0. The membrane-forming solution contained 33 mg/ml phosphatidylethanolamine (bovine brain) and 13 mg/ml phosphatidylserine (bovine brain) in decane. The phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL); decane was repurified before use by passage over neutral alumina. Saxitoxin (paralytic shellfish poison standard) was obtained from the Food and Drug Administration (Cincinnati, OH); BTX was a gift from Dr. John Daly (NIAMDD, Bethesda, MD).

Preparation of Membrane Vesicles

Membrane vesicles were prepared from rat brain as described by Krueger et al. (1979). Briefly, rat forebrains were homogenized in isotonic sucrose using a cavitating tissue disrupter (Ultraturrax, Tekmar Co., Cincinnati, OH) and the homogenate was subjected to differential centrifugation. The 1,000 *g* and the 10,000 *g* pellets were discarded and the 100,000 *g* pellet (P_3) was resuspended in 0.4 M sucrose at 10–20 mg protein/ml. Aliquots of P_3 were stored at −77° for up to six months without loss of activity. This fraction was enriched about fivefold in ^{3}H-STX binding sites as compared to the crude brain homogenate (Krueger et al., 1979).

Incorporation of Sodium Channels

Incorporation of sodium channels was accomplished using the "fusion" technique (Miller, 1978; Cohen et al. 1980; 1982). The membrane vesicle suspension (100–500 ng/ml protein) was added to the *cis* side of a preformed planar bilayer and BTX (600 nM) was added to the *trans* side. The conductance of the membrane increased in steps, each reflecting the incorporation of one to several single sodium channels (Krueger et al., 1983). Following incorporation of the desired number of channels (normally one to five), the *cis* side was perfused with vesicle-free solution. STX was added to the *cis* side from a 1,000-fold concentrated stock solution.

Data Acquisition and Analysis

The current across the bilayer was measured and command voltages applied to the bathing solutions via a pair of Ag/AgCl electrodes. The side to which brain membrane vesicles were added was designated the *cis* side; the opposite (*trans*) side was held at virtual ground. Prior to addition of biological material, the conductance of the bilayer was <20 pS (~10^{-6} S/cm^2) and the capacitance was ~0.5 μF/cm^2. Two current-to-voltage converters were used in these studies, one having a bandwith of ~80 Hz and the other ~500 Hz. Similar results for the steady-state voltage dependence of gating of BTX-activated sodium channels, in the absence of STX, were obtained with each of these converters. The faster amplifier was built using an LF157H operational amplifier (National Semiconductor, Santa Clara, CA), and a 10^9 (± 1%) Ω feedback resistor (Eltec model 102). Data were recorded continously using an FM tape recorder (Vetter, model B) during an experiment, at or near the bandwidth of the current-to-voltage converter. Subsequently, the data were digitized in 4,096 point-segments at 1, 2 or 5 ms per point using a Nicolet 2090-3A digital oscilloscope, and the digitized records were stored on floppy disks. Data transcription and analysis were controlled by a microcomputer (Micro II, Plessey Peripheral Systems, Irvine, CA) based on an LSI-11/2 processor (Digital Equipment Corporation, Maynard, MA). Software for data handling and analysis was developed using the interpretive language of DAOS (Data Analysis Operating System, Laboratory Software Associates, Melbourne, Australia).

For analysis of STX-induced current fluctuations, the records were filtered (low-pass) with a corner frequency of 30 Hz using an eight-pole Bessel filter (902 LPF 1B, Frequency Devices, Haverhill, MA). This eliminated most of the brief closing fluctuations caused by the voltage-dependent channel gating process, leaving visible the longer events which were primarily due to STX block and unblock. Even at a bandwidth of 500 Hz, many of the visible transitions resulting from the voltage-dependent channel gating were incompletely resolved. We have not attempted a dwell-time analysis of these fluctuations.

All records were visually monitored on a CRT display during analysis. Limiting current levels used in determining the fractional open times, and discriminator levels for the dwell-time analyses, were checked against a whole group of records before carrying out the analysis. If necessary, the selected values were changed to accommodate small shifts in the baseline within a series of records. Also, all analyses were oriented toward the interval, usually the whole record, demarcated by two vertical cursors. When necessary, we excluded from the analysis abnormally noisy segments of records—for example, occasional periods of high frequency, nonquantal fluctuations possibly caused by partial, reversible, breakdown of the membrane.

Fractional open times, resulting from voltage-dependent channel gating and from STX block of the channels, were determined independent of the dwell-time analysis. The background leakage current, i_L, across the membrane, with all incorporated channels closed or blocked, was determined either by setting a horizontal cursor at the low conductance edge of the envelope of these fluctuation, or by averaging cursor-selected, long, well-defined events during which no channels were conducting. The maximum current, i_{max}, (all channels conducting) was determined in an analogous manner. The average current, $\langle i \rangle$, over a long stretch of record (averaging ~20 s for gating, or ~170 s when studying STX-block) was determined. The fractional open time, f_o is then given by

$$f_o = (\langle i \rangle - i_L)/(i_{max} - i_L) \qquad (1)$$

and the fractional closed or fractional blocked time is $(1 - f_o)$.

Dwell-time distributions were determined, one conductance level at a time, by scanning records for periods when the current fell in a target zone between two discriminator levels. These discriminator levels were centered between the conductance level of interest and the adjacent ones on either side. The program was based on a digital triggering routine which recognized positive or negative slope transitions into or out of the target zone. Dwell times were scored as a density function that recorded the number of occurrences at each possible dwell time. After scanning a complete set of records, cummulative distribution functions were calculated (see Fig. 7) to display the total number of events lasting at least as long as any given time, t. If a conductance level corresponds to a single, time-homogeneous, Markovian kinetic state (e.g., Colquhoun and Hawkes, 1981; French and Horn, 1983), these distributions should be a single exponential with a mean value equal to the time constant for decay of the exponential. In fact, for 19 calculated distributions, based on an average of 126 events each, the time constants, τ, were linearly dependent on the mean dwell times, $\langle t \rangle$ (correlation coefficient, $r = 0.97$) with a slope of 1.3. Thus, the distributions were approximately, but not perfectly, exponential, with more short events than expected. These additional events may have been due to the transient, partial breakdown of the membrane already mentioned, or to some infrequently occupied, poorly resolved, and short-lived kinetic state. We cannot choose between these alternatives. In the Results and Discussion sections that follow, we define the characteristic dwell-time constant, τ, for a distribution as the value obtained by a linear least-squares fit of the cumulative distribution to the function

$$\ln N(t) = \ln N_T - t/\tau \qquad (2)$$

where $N(t)$ is the number of events with a lifetime of at least t, and N_T is the total number of observations. The fits were performed over the decline of the distributions from N_T to a value of ~0.05 N_T. (For each of the 19 log-transformed cumulative distributions, $r > 0.97$.)

Voltage Convention

In this paper we have expressed all voltages in the normal cellular physiological convention (inside minus outside) on the assumption that STX acts only from the extracellular side. This is the reverse of the convention, $E_m = E(cis) - E(trans)$, used in a number of planar bilayer publications and by Krueger et al. (1983).

RESULTS

Single-Channel Current Fluctuations Due to Gating and Due to STX Block

Fig. 1 illustrates the two temporally distinct populations of current fluctuations through sodium channels in bilayers when both BTX and STX are present. The first of these consists of brief closing events that generally last for periods of a few tens of milliseconds or less, and appears to include many events that are too brief to be completely resolved at the bandwidth of our recordings (cf. Quandt and Narahashi, 1982). When viewed at sufficiently high resolution, many other fluctuations are seen as roughly square current steps, which consistently correspond to a unit conductance of ~30 pS in 500 mM NaCl (see Fig. 3). This population of brief closures, from a well-defined "open" current level, can be seen in both the absence and the presence of STX (see upper and lower records in Fig. 1). We presume that these relatively rapid fluctuations are due to the voltage-dependent gating of the BTX-activated sodium channels. The steady-state voltage dependence will be discussed in more detail below. Observations on numerous preparations appear to show a slight but systematic variation in the kinetics of the fluctuations when the voltage was changed from −60 mV to +60 mV. At positive potentials, closings were slightly less frequent, but tended to be somewhat longer in duration. However, over this entire range, the channels remained open almost all (>98%) of the time. This point will become important in our analysis of the voltage dependence of STX block.

Examining the lower section of Fig. 1, it is immediately obvious that a number of "closing" events of much longer duration appear after addition of STX. In fact, "closing" of the channels becomes much more probable and, at −60 mV, there are several intervals during which neither channel is in the conducting state. These long periods during which one or both channels are "closed" occur only in the presence of STX, and hence it is reasonable to conclude that they are caused by STX block of the channels rather than by closing of the channel gates. This conclusion is also supported by the observation that the probability of entering one of these long-lived "closed" states increases proportionately as the STX concentration is increased (Krueger et al., 1983, and see below).

Fig. 1 illustrates two further points. At +60 mV, with 1 nM STX present, no particularly long-lived "closing" step occurs. Immediately after the voltage change to −60 mV, ~30 s follow in which there is still no extended "closure." Both channels remain open for most of this time. At longer times, however, there are continuous stochastic current fluctuations including periods during which both, one, or neither channel is blocked.[1] (Only the 0-channel and 1-channel levels are shown after the initial blocking event in Fig. 1, lower trace.) Thus, the lower record in Fig. 1 hints at two conclusions: that negative voltages favor block by STX, and that it takes many seconds to establish a new stationary-state following a step in voltage at this STX concentration.

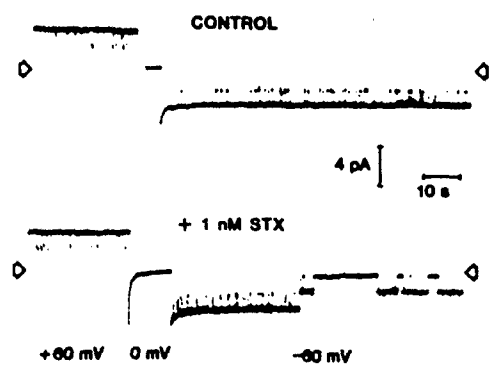

FIGURE 1 Current fluctuations from a membrane containing two BTX-activated sodium channels. A downward deflection in the current record represents an inward (*cis* to *trans*) current across the bilayer. Zero current levels are shown by the arrows. *Top trace*: control record. The channels remained open almost all of the time, with brief closings towards the zero-current level. These closings were presumably due to the voltage-dependent gating of the channels. The +60 mV segment was actually recorded after the −60 mV segment, but is shown here preceding the −60 mV segment to facilitate comparison with the lower record taken in the presence of STX. Capacity transients are limited by the range of the pen sweep. *Lower trace:* record taken after addition of 1 nM STX to the *cis* side. The long closing steps, including an extended period during which neither channel was conducting, were most likely due to block of individual channels resulting from the binding of STX. Records were filtered (low pass) at 60 Hz on playback.

Dependence of the Open and Blocked Dwell Times on STX Concentration

If we are to establish that the slower population of fluctuations, just described, are caused by STX binding to and dissociating from the channels, two criteria should be met. First, the lifetime of the blocked state should depend only on the intrinsic rate of dissociation of the blocker from the channel, and hence should be independent of STX concentration. Second, for any given channel, the lifetime of the open state will depend on the rate of binding of the toxin, which, for a 1:1 stoichiometry, would be directly proportional to the STX concentration. This prediction may be generalized to apply to a membrane containing several channels using the birth-death analysis applied by Labarca et al., (1980). With the additional assumption that channels present in the membrane are blocked independently of

[1] In Fig. 1 (bottom trace) the time spent at the two-channel level (neither channel blocked) prior to the long "closure" after changing to −60 mV is about equal to the mean dwell time (28 s) predicted by the rate constant for STX block determined later in the paper. The calculated probability that both channels would remain unblocked for at least 30 s following the voltage step to −60 mV is $p = 0.34$.

one another, one predicts the following relations

$$1/\tau_o = n\lambda \qquad (3)$$

where τ_o represents the characteristic dwell-time constant from the distribution of "zero-channel–open" dwell times, n is the number of channels in the membrane ($n = 7$ for the experiment that was analyzed in detail for this paper), and λ is the rate of dissociation of STX from a single channel (in s^{-1}). In addition,

$$1/\tau_k = (n - k)\lambda + k\beta[\mathrm{STX}]. \qquad (4)$$

Here, τ_k is the characteristic dwell time in the current or conductance level for which k channels are open, and β is the rate constant, in $M^{-1}\,s^{-1}$, for binding of STX to the channel. For the one-channel level, as one approaches conditions under which all channels are blocked, $1/\tau_1$ approaches $\beta[\mathrm{STX}]$.

In Fig. 2, we plot the characteristic reciprocal dwell times against [STX] at +58 mV, for the zero- and one-channel levels. The value of $1/\tau_1$ increases linearly with [STX] (slope = $1.1 \times 10^6\,M^{-1}\,s^{-1}$), while on the same scale, $1/\tau_o$ is essentially independent of STX concentration ($r = -0.28$). The zero intercepts of the regression lines do not differ significantly from the predictions of Eqs. 3 and 4, given the scatter in the data. Thus, the fluctuation kinetics are consistent with the commonly accepted 1:1 stoichiometry for STX binding to the channels.

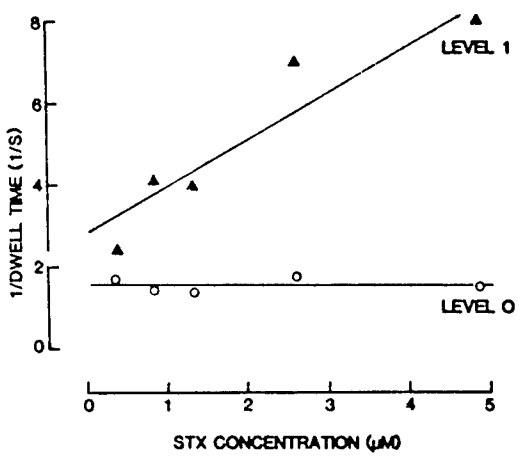

FIGURE 2 Dependence of the reciprocal dwell times on the STX concentration. Dwell times at each concentration were estimated as described in Materials and Methods. The reciprocal dwell time in level 0 (no channels open) is determined by the rate of STX unbinding from the channel, and is independent of STX concentration. The reciprocal dwell time in level 1 (one channel conducting) is primarily determined by the rate of STX binding, and is linearly dependent on STX concentration. Lines shown are linear least-square fits to the data. For level 1, slope = $1.1 \times 10^6\,M^{-1}s^{-1}$ and the correlation coefficient, $r = 0.93$. For level 0, $r = -0.28$. $E = +58$ mV.

Voltage-dependence of Gating of BTX-activated Sodium Channels

The data presented in Figs. 3 and 4 show how a channel's intrinsic, voltage-dependent gating determines the probability that a channel will be found in the open state. In the sample current records at −70 mV (Fig. 3, right), presented on a time scale expanded about 20-fold with respect to those of Fig. 1, one can see only a few closing fluctuations in the current record. Although at −70 mV most are too brief to be completely resolved, many more closing fluctuations are visible as the voltage is further hyperpolarized to −80 mV, and at −90 mV the channel flickers rapidly between open and closed states, spending an almost equal fraction of the time in each. At −100 mV the channel is closed ~3/4 of the time. A parallel illustration of this voltage-dependence appears in the frequency-amplitude histograms, in which the number of data points at each conductance level is plotted against conductance.

In Fig. 4, we have defined the operational range and steepness of this voltage-dependence by fitting the fractional open time, f_∞ to a Boltzmann function of the voltage, E:

$$f_\infty = 1/\{1 + \exp(qF[E - E_{0.5}]/RT)\}. \qquad (5)$$

The precise values taken by this function are determined by the parameters q, the apparent gating charge, and $E_{0.5}$, the voltage at which $f_o = 0.5$. The value of q determines the slope of the curve and $E_{0.5}$ is the midpoint of the range of voltages over which f_o changes. Actual values of these parameters were obtained by a linear least-squares fit to the following function derived by rearranging Eq. 5.

$$\ln([1 - f_o]/f_o) = qF(E - E_{0.5})/RT. \qquad (6)$$

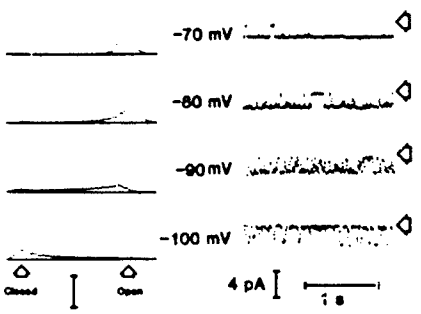

FIGURE 3 Voltage dependence of gating of a single, BTX-activated sodium channel. Histograms of frequency of occurrence vs. conductance (*left*), and sample digitized records (*right*) are shown. A downward deflection in the current record represents an inward (*cis* to *trans*) current across the bilayer. The arrow on the right side of each current record indicates the zero-current level. The vertical bar at the bottom of the *left* panel indicates 500 points for the histograms; the spacing between the arrows below the histograms is 30 pS. Records were filtered at 80 Hz on playback from an FM recorder. In the range of −70 mV to −100 mV, the channel goes from spending most of the time in the open state (−70 mV) to spending about 3/4 of the time in the closed state (−100 mV).

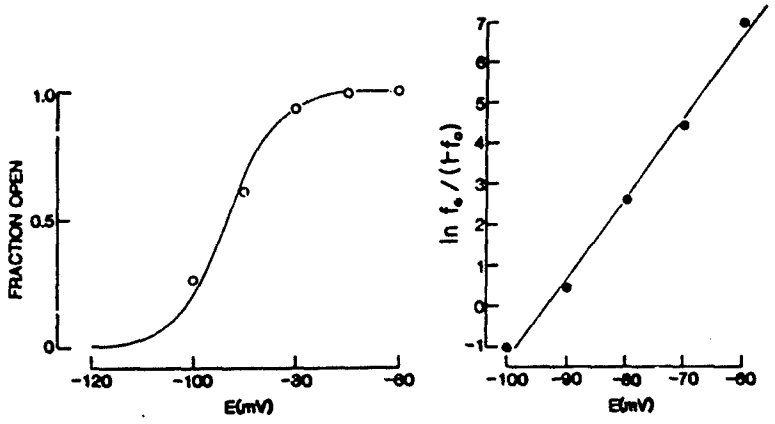

FIGURE 4 Voltage-dependence of channel gating. The fractional open time (f_o) is plotted vs. voltage (*left*) and the same data are plotted in linearized form, $\ln[f_o/(1-f_o)]$ vs. E, on the right. The linearized data were fit by the assumption that the channel spends 50% of its time open at $E_{0.5} = -93$ mV, and that there is a gating charge of $5e^-$/channel. Further details appear in the text. Data are from the same membrane as those shown in Fig. 3.

The values obtained—an apparent gating charge of 4–6 electronic charges/channel (5.6 ± 1.0, SE, three experiments), and a half-open voltage of $E = -93$ mV (-93 ± 5 mV, SE, three experiments)—are close to those determined from macroscopic current measurements on BTX-activated sodium channels in voltage-clamped neuroblastoma cells (Huang et al., 1982). The key point to be made for the purposes of this paper is that the probability of the channel gates being found open changes significantly with transmembrane potential only over a restricted range of voltages that is more negative (hyperpolarized) than −60 mV.[2]

Saxitoxin Block is Voltage-dependent in a Range Where the Channel Gates Remain Open

The remaining results are based on long records taken in the presence of STX from a membrane containing seven sodium channels. It can be seen from Fig. 5 that all channels are blocked almost all of the time by 320 nM STX at −60 mV, whereas, at +58 mV, up to three, and occasionally four, of the seven channels in the membrane were open simultaneously. At these voltages, channels remained open >98% of the time in the absence of STX (see control record, Figs. 1 and 3). Block by STX thus appears to be voltage-dependent. We have observed, without exception, qualitatively similar voltage dependence in each of six experiments.

To examine the voltage dependence of the steady-state fractional block quantitatively, we determined, from extended recordings, the mean current, corrected for the conductance of the bilayer with no channel conducting. The fraction blocked, f_b, was then estimated as

$$f_b = 1 - f_o \qquad (7)$$

where f_o is the fractional open time defined by Eq. 1. Plots of $1/f_b$ vs. $1/[\text{STX}]$ were then used to estimate the apparent dissociation constants, at these voltages, for the

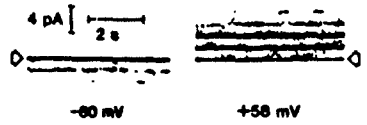

FIGURE 5 Voltage dependence of current fluctuations due to blocking and unblocking of the sodium channels by STX. Current traces from a membrane containing seven sodium channels, with 320 nM STX on the *cis* side, are shown. A downward deflection in the current record represents an inward (*cis* to *trans*) current across the bilayer. At −60 mV, 16 traces are superimposed; at +58 mV, 24 traces are superimposed. The records were filtered (lowpass) at 20 Hz on playback from FM tape. The common level, indicated by the arrows, corresponds to zero current, and the current increment between adjacent levels is ~1.8 pA. Notice that there were never more than two channels simultaneously open at −60 mV, and never more than four open at +58 mV, even though control records showed current corresponding to seven channels. The voltage dependence seen here must be due to a voltage dependence of STX block since the channels remain open essentially all of the time at these voltages in the absence of STX (see control trace, Fig. 1).

[2]Although we have no direct knowledge of either the resting potential in the cells from which our channels originated, or the normal conductance-voltage relation for these channels in the absence of BTX, it seems likely that, as in cellular preparations, BTX positions the conductance-voltage curve at more hyperpolarized potentials than normal. Our ionic conditions, which were chosen to facilitate channel incorporation and to provide a reasonable signal-to-noise ratio, include higher than physiological monovalent ion concentrations and internal (*trans*) calcium, and lower than physiological external (*cis*) calcium. Millimolar concentrations of internal calcium had little or no effect on sodium conductance-voltage relations in squid axon (Begenisich and Lynch, 1975). Lowered external calcium generally shifts conductance-voltage relations in the hyperpolarizing direction (Frankenhauser and Hodgkin, 1957; Hille et al., 1975), but the magnitude of this shift would be reduced by the high monovalent ion concentration (Hille et al., 1975). These data suggest that our ionic conditions might produce a negative shift in the f_o vs. E relation, on the order of 10 mV.

STX block from the equation

$$1/f_b(E) = 1 + K_d(E)/[\text{STX}]. \quad (8)$$

At $E = -60$ mV, we had sufficient data to estimate f_b at only two STX concentrations. In that case we made two discrete estimates of $K_d(-60)$ by substituting $f_b(-60)$ and [STX] directly into Eq. 8 and solving for $K_d(-60\text{ mV})$ (Fig. 9, ○).

As indicated by the increasing slopes of the reciprocal plots in Fig. 6, $K_d(E)$ increases with increasing voltage, a quantitative expression of the observation noted earlier that the degree of block was reduced at positive voltages (Fig. 5). The actual dependence of K_d on E is shown in Fig. 9, where we compare these estimates from steady-state measurements (open symbols) with those from independent kinetic determinations (●).

Voltage Dependence of the Apparent Binding and Dissociation Rates

A marked decrease in the dwell time for the single-channel open level, produced by changing the voltage from +58 mV to −60 mV, is clearly apparent in Fig. 7. This indicates an increase in the binding rate for STX over this range. In Fig. 8, we show the rate constants for binding (blocking), β, and dissociation, λ, calculated from Eqs. 3 and 4, plotted semilogarithmically against voltage. The voltage dependence shown by the two rates is complementary, β increasing and λ decreasing as E is made more negative. The regression lines for the semilogarithmic plots in Fig. 8 suggest that in each of the binding and dissociation steps a single, elementary charge responds to ~30% of the transmembrane voltage or, equivalently, two charges sense 15%. Negative voltages, which attract the cationic toxin toward the inside of the membrane, speed toxin binding and slow its dissociation. Quantitatively, for the data in Fig. 8, this information is expressed in the following relations:

$$\lambda_E = \lambda_0 \exp(0.28\, FE/RT) \quad (9)$$

$$\beta_E = \beta_0 \exp(-0.35\, FE/RT) \quad (10)$$

where $\lambda_0 = 0.095\ s^{-1}$ and $\beta_0 = 0.935 \times 10^7\ M^{-1}\ s^{-1}$. The value obtained here for β at $E = 58$ mV ($0.38 \times 10^7\ M^{-1}\ s^{-1}$) may be directly compared with the slope (1.1×10^7

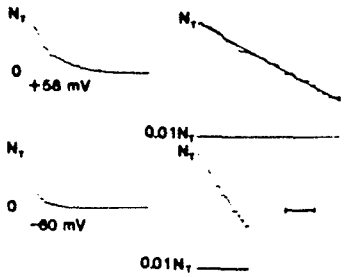

FIGURE 7 Voltage dependence of open-state dwell times. Cumulative dwell time distributions are shown, for $E = +58$ mV and -60 mV for the one-channel–open conductance level, from the same membrane as for Fig. 5. The abscissa represents time, and the ordinate represents the number of events that lasted at least as long as the time, t. *Left*, complete distributions using a linear scale on the ordinate; *right*, semilogarithmic plots of the same data. N_T, the total number of events observed at each potential, was 58 at +58 mV and 75 at −60 mV. The straight lines through the data are linear regression lines through the log-transformed data. In the left-hand parts of the figure, the vertical cursors indicate the segment of the distribution that was used for the fits shown on the right. Notice the marked decrease in the open (unblocked) state dwell times when the voltage is changed from +58 mV to −60 mV. Data were collected in the presence of 320 nM STX. The time scale bar represents 1 s (*left* frame) and 0.5 s (*right* frame).

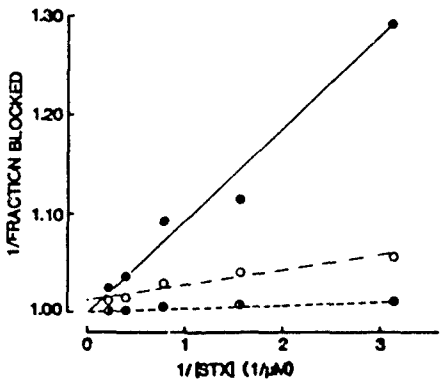

FIGURE 6 Reciprocal plots of dose-response data for STX block at three different voltages. The reciprocal of the fractional block of the sodium channel current is plotted vs. the reciprocal of the STX concentration, with linear regression lines ($r > 0.90$ in each case) for each set of data. Data are from the same membrane as that in Fig. 5. Points shown are for $E = +58$ mV (●), +30 mV (○) and for −30 mV (half-filled circles). Apparent dissociation constants derived from these data are plotted as a function of voltage in Fig. 9.

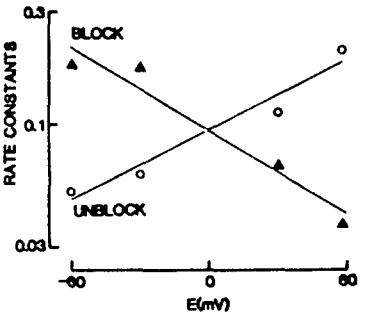

FIGURE 8 Dependence on voltage of the apparent binding and dissociation rate constants for STX block of the channels. The rate constants were derived from the reciprocal dwell times under the assumption that each of the seven channels in the membrane could be independently blocked by STX. The lines shown are linear least-square fits to the logarithm of the rate constants (correlation coefficients, $r = 0.98$ in each case). Notice that the blocking and unblocking rate constants show a voltage dependence of approximately equal steepness but of opposite sign, such that hyperpolarization speeds blocking and slows unblocking. Units on the ordinate are s^{-1} for unblock and $10^8\ M^{-1}\ s^{-1}$ for block. Further details of the analysis are in the text. The rate constants were determined from dwell times measured in the presence of 320 nM STX.

$M^{-1}s^{-1}$) of the plot of reciprocal open dwell times vs. STX concentration in Fig. 2. Part of this discrepancy can be explained by the filtering of the records prior to analysis. As the STX concentration increases, consequently decreasing the open (unblocked) dwell time, the filtering obscures a higher and higher fraction of the open (unblocked) events. At the highest STX concentration used in Fig. 2, up to 10%–20% of the unblocking events would be expected to go unrecognized.

The apparent dissociation constant $K_d(E) = \lambda_E/\beta_E$ may be calculated from these data, and the derived estimates agree closely with those obtained from the steady-state block measurements mentioned earlier (compare open and filled symbols in Fig. 9). In the context of a model in which STX must enter the membrane field (cf. Woodhull, 1973), $K_d(E) = \exp(z\delta FE/RT)$, where z is the valence ($z = 2$ for STX) and δ is the fraction of the transmembrane voltage sensed by the toxin. When $K_d(E)$, calculated from the blocking and unblocking rate constants, was plotted against membrane potential, we determined a value for $z\delta$ of 0.63 ($\delta = 0.3$), suggesting that the divalent toxin senses ~30% of the transmembrane field when bound in the steady state. When K_d, determined from our steady-state fractional block measurements, was plotted against potential, we obtained a value for δ of ~0.4. Hence, 30–40% of the voltage affects toxin binding, either directly or indirectly.

DISCUSSION

Hille (1968), in describing the block of sodium channel currents in frog node by STX, wrote "... amplitudes of the peak sodium currents were reduced by the same fraction at every voltage, whether the current is inward (negative) or outward." That study, and others (for reviews, see Narahashi, 1974; Ritchie and Rogart, 1977) strongly suggested that one toxin molecule bound to each channel to produce block. Subsequently, kinetic studies (Schwarz et al., 1973) showed that equilibration time constants for TTX—a toxin showing comparable binding affinity, block, and unblock following a step in toxin concentration—were on the order of tens of seconds at concentrations near the dissociation constant. It became clear that measurements of peak sodium current during a depolarizing voltage step from a fixed potential would assay the degree of block for the steady state reached at the holding potential prior to the pulse. Later studies by Almers and Levinson (1975), Ulbricht and Wagner (1975 *a*, *b*), and Cohen et al. (1981) examined the effect on TTX block of steady depolarization preceding a test pulse. In addition, Krueger et al. (1979) examined the effect of potassium-induced depolarization on ^{3}H-STX binding to rat brain membrane vesicles. All of these studies appeared to support the earlier conclusion that block by STX was due to voltage-independent binding with a 1:1 stoichiometry.[3] The question then arises, why should the toxin block appear to be voltage-dependent for BTX-activated sodium channels incorporated into bilayers?

There are at least three explanations for this voltage-dependent block by STX. The voltage dependence that we observed may simply have been overlooked because of the length of the equilibration times compared with the usual duration of voltage clamp pulses. The macroscopic relaxation time constant can be calculated from the binding and dissociation rate constants (Fig. 8) using the expression

$$\tau_{rel} = 1/(\lambda + \beta[\text{STX}]). \qquad (11)$$

Using the rate constants for $E = 0$ mV, $\tau_{rel} = 5.3$ s when [STX] = 10 nM. Although the long relaxation times for TTX and STX block were not taken into account in some earlier studies, this first possibility can probably be discarded on the basis of the careful studies of Ulbricht and Wagner (1975 *a*, *b*), Almers and Levinson (1975), and Cohen et al. (1981).

The second possibility is that BTX modification causes the toxin block to become voltage-dependent. This possibility is of interest in light of the observation that BTX induces changes in the ion selectivity of sodium channels (see Khodorov, 1978, for a brief review). To date, we have been unable to address this question in our preparation, since with the relatively large bilayers used in these studies we lacked the necessary current-time resolution to study

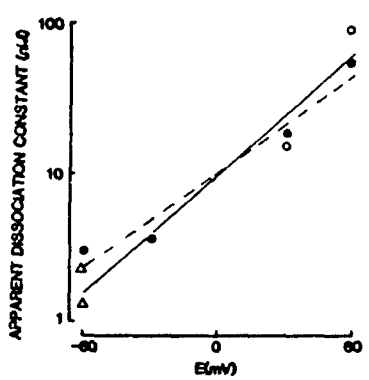

FIGURE 9 Apparent dissociation constants for STX block plotted vs. transmembrane voltage. Two independent determinations are shown. Open symbols (triangles and circles) were obtained from fractional blocked times. The open circles were obtained from the slopes of the regression lines in Fig. 6. Since we only determined the fractional block at two STX concentrations at −60 mV, the two individual points are plotted here (△); the mean of these two points was used, with the open circles, to calculate the linear regression line (—). The solid symbols and the dashed line (---) are the apparent dissociation constants, and the corresponding fit, obtained from the rate constants estimated from the dwell times (see Figs. 7 and 8). The half-filled circle represents the superposition of an open and a solid circle.

[3] A report by Baer et al. (1976) that depolarization enhances TTX block of cardiac sodium channels has been challenged on technical grounds (Cohen and Strichartz, 1977; Cohen et al., 1981) and is not supported by more recent voltage clamp studies (Colatsky and Gadsby, 1980; Cohen et al. 1981).

sodium-channel currents in the absence of BTX. However, published reports suggest that both binding of ^{3}H-STX (Krueger et al., 1979) and block of sodium channels by TTX (Mozhayeva et al., 1982) are unaffected by BTX activation.

The third possibility is that one of our experimental conditions either induces or accentuates the voltage dependence. Specifically, we used calcium and sodium at the physiological inner surface of the channels in concentrations about 100-fold higher than those found in intact nerve. Binding of a cation to a site accessible from the inside, within the transmembrane field, might repel the cationic toxin from a binding site at the outer surface, and would thus confer voltage-dependence on the block, with the orientation that we observed. At concentrations far below their dissociation constants, binding of the "competing" ions would be negligible regardless of voltage, hence voltage would have no significant effect on STX binding. This mechanism is related to the "modified competition" proposed by Ulbricht and Wagner (1975 *a*) to account for an apparent voltage dependence of TTX binding seen only at low pH. The third possiblity could be tested by varying the composition of the solution at the inner surface of the channels (the *trans* side). Specifically, if STX binding (which may be intrinsically voltage-independent) can be prevented or reduced by the occupancy of a cation binding site accessible from the inside and within the membrane field (i.e., occupancy of that site would be voltage-dependent), then the apparent voltage dependence of STX block should be reduced by lowering the sodium and/or calcium concentrations on the inside of the membrane. We are now performing experiments to test this possibility.

With regard to the specific molecular basis of the voltage dependence, it will also be of interest to examine the effect of voltage on TTX block of these channels, since TTX is monovalent at physiological pH, while STX is divalent. If these blockers enter the transmembrane electric field to block the channels, one might expect the block by STX to be more strongly influenced by voltage. Nonetheless, the experiments may not necessarily lead to an unambiguous conclusion, as the effect of voltage on the action of divalent cations with two separated charges can show a complex dependence on their size and molecular structure (Miller, 1982). The larger size of STX (the charges are separated by ~3 Å) makes it possible that each of the charged groups would penetrate a different fraction of the transmembrane voltage.

Two observations have been made in other systems, from which it appears that depolarization may be able to reduce the degree of TTX block. In cardiac muscle, a slight reduction, by depolarization, of block of background sodium conductance is shown in Fig. 1 *C* of Colatsky and Gadsby (1980). This parallels our own observations, but the effect is less marked. Although Mozhayeva et al. (1982) were addressing an apparently unrelated phenomenon (viz., an irreversible effect of TTX on gating of BTX-activated sodium channels), their records (Fig. 1 *C*) show a slow, steady increase in current amplitude toward the end of 50–100-ms depolarizing pulses. This could be the result of slowly relaxing, depolarization-induced release of TTX block of the BTX-activated channels.

We thank Carl Phillips for preparing the figures, G. Ay for fabricating the chambers, J. Michael and T. Sparks for assisting with the electronics, and Brian Hall for help installing an updated version of DAOS software. We are expecially indebted to Dr. J. Daly for providing a generous supply of BTX for these studies. We also thank Drs. M. S. Brodwick, M. D. Cahalan, D. C. Eaton, L. Goldman, P. A. Pappone, R. Payne, J. J. Shoukimas, G. Strichartz, and R. W. Tsien for helpful discussions and comments.

This work was supported by National Institutes of Health grants NS-16775 to R. J. French and NS-16285 to B. K. Krueger, by the Bressler Foundation of the University of Maryland School of Medicine, and by contract DAMD-17-C-2188 from the U.S. Army Medical Research and Development Command, both awarded to B. K. Krueger and R. J. French.

Received for publication 2 May 1983 and in final form 11 June 1983.

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DISCUSSION

Session Chairman: Alan Finkelstein *Scribes*: Lawrence B. Weiss, Juli Lai Weiss, and Charles L. Bowman

PAPPONE: In your paper you give two possible explanations for the voltage dependence of STX block of BTX-modified Na^+ channels. It could be an effect of the BTX modification or of the unphysiologic Na^+ concentration on the *trans* side. Do you have any data which address this question?

FRENCH: These may be two distinct issues. Firstly, is BTX modification required for the voltage dependence? Secondly, when voltage dependence occurs, is it due to an interaction between STX and some "internal" ion? At this point, we have not done experiments at wide enough bandwidths to study the channels in the absence of BTX and thus resolve the question of primary BTX effect. Krueger et al. have addressed this in K^+-depolarized synaptosomes and found no effect of BTX on STX binding. This question is raised for our system because the Na^+ concentration on the *trans* side was orders of magnitude higher than physiologic concentration. Therefore, the possibility is raised that Na^+ may displace BTX. We have no direct evidence for this.

PAPPONE: Because you have interpreted the voltage dependence of block by STX to mean that STX has moved into the channel and because STX and TTX have different numbers of charges, have you compared the voltage dependence of these two toxins?

FRENCH: Yes. We have some preliminary results and have found that TTX exhibits the same voltage dependence as STX.

FINKELSTEIN: Is it correct that for both STX and TTX there is an *e*-fold change in block for 40 mV?

FRENCH: Yes.

RUBINSON: I would argue that it is something other then the movement of the next charge on the toxin in the membrane field that is causing the effect.

FRENCH: I would agree.

FINKELSTEIN: Could you clarify your point about the experimental Na^+ concentrations?

FRENCH: We used symmetric 0.5 M NaCl in our experiments.

ANDERSEN: In collaboration with Bill Green and Larry Weiss, I have done similar experiments with dog brain synaptosomes. Barring any major species difference, we have encountered the same voltage dependence for TTX binding to BTX-modified Na^+ channels. This voltage dependence is comparable with that reported here and is independent of Na^+ concentration. We have also found the K_D for TTX block at a given potential varies with Na^+ concentration. Between 50 mM and 2.5 M NaCl there is no change in the voltage dependence of TTX block and there appears to be a competitive interaction between Na^+ concentration and TTX block. This raises the question of whether or not TTX and STX bind in the permeation pathway. Further, although the block induced by STX and TTX are voltage dependent, we don't know if the binding of these toxins is voltage dependent or if the block represents an independent process. Is there any evidence whether these neurotoxins bind in the permeation pathway or whether they bind at an allosteric site?

FRENCH: There is no direct evidence. The idea that STX and TTX may act as a plug is suggested by the fact that these toxins contain guanidinium groups, and that guanidinium can carry current through the Na^+ channel.

YEH: The results of our (Tanguy, Narahashi, and Yeh, unpublished) experiment with BTX on Na^+ channels in squid axon membranes support the idea that BTX modification causes the toxin (STX) block to become voltage-dependent. As had been reported in many other preparations, BTX has two major effects on the gating kinetics in squid axon; it opens Na^+ channels at very negative potentials and it removes Na^+ inactivation. In addition, BTX-modified Na^+ channels have different pharmacological profiles. For example, in the presence of 10 nM STX, the Na^+ current at −80 mV was suppressed to ~50% of the control, whereas at +20 mV, the

suppression was <20%. Thus the blocking action of STX in BTX-modified channels is voltage dependent.

Furthermore, this voltage-dependent block occurs very rapidly, as reflected in the lack of time dependence of the unblocking rates. This is in marked contrast to the effect of STX on normal Na channels. As French pointed out, STX block of normal Na^+ channels is not voltage dependent and the unblocking rate is rather slow.

MOCZYDLOWSKI: In Chris Miller's lab, we have been studying Na^+ channels from rat muscle in bilayers and our data confirm that of Olaf Andersen and Robert French with respect to the voltage dependence of toxin binding. In collaboration with Gary Strichartz and Sherwood Hall, we have studied six different derivatives of STX in which the net charge is modified by sulfate groups. The various toxins bear a net charge from 0 to +2. All these toxins exhibit the same voltage dependence. Our findings on Na^+ competition reveal only an effect on the association rate with no effect on the off rate constant in the range of 40–600 mM Na^+. In the simple competitive model, this suggests that the binding constant for Na^+ is voltage independent while toxin binding to this site is voltage dependent.

FRENCH: Those observations argue against the voltage dependence being due to Na^+ from the inner side because this would probably modify the off rate.

ANDERSEN: We find no change in the off rate constant from 50 mM to 2.5 M Na^+, making it unlikely that there is an effect of Na^+ from the inner side. There is, however, a flaw in this argument. If there is a 1.0 mM K_D for Na^+ to an inner binding site, we may really be looking at a Na^+–TTX interaction in a doubly occupied channel.

HALL: Because your experiments are done in decane-containing membranes which exhibit electrostrictive thinning, can you be sure that this deformation is not the origin of your voltage dependence?

MOCZYDLOWSKI: We have done experiments in folded membranes without decane, and our results are the same.

BENNETT: If the toxin site is first in the permeation pathway and then in the blocked state, the entire potential might drop across the single guanidinium group, independent of the rest of the molecule.

FRENCH: Such a potential profile offers a possible way of explaining the steep voltage dependence of action of the bulky toxin molecules, but it also raises two questions. Why don't we see a voltage dependence corresponding to the whole voltage drop? And how would this picture apply to the more complex derivatives that Ed Moczydlowski and Chris Miller have been studying?

MOCZYDLOWSKI: What you're proposing is that only one guanidinium is entering the field, while the second guanidinium would be completely excluded even though the distance between these two groups is 3–4 Å for the saxitoxin molecule. This explanation seems unlikely.

FINKELSTEIN: To rephrase this important question: in the simple blocking models it has been assumed that the electric field is the same in the blocked and unblocked states, but this model may not be correct. It may be more correct to ask, what is the voltage profile that the blocking ion sees when the site is unblocked? And what does it see after the site is blocked?

BARCHI: Is the evidence you have presented consistent with a very small voltage-dependent change in tertiary or quarternary structure in the binding site itself?

FRENCH: Yes.

ANDERSEN: I would like to point out another very interesting finding, by Almers and Levinson (1975. *J. Physiol.* (*Lond.*). 247:483), which is usually cited as evidence for voltage-independent block but which actually supports voltage-dependent block. In a muscle preparation in normal NaCl they determined a K_D for TTX binding. These muscles were then K^+ depolarized in a Na^+-free solution and the K_D was unchanged. However, other investigators (Reed and Raftery. 1976. *Biochemistry.* 15:944; Barchi and Weigele. 1979. *J. Physiol.* (*Lond.*). 295:383) as well as ourselves, have demonstrated Na^+ competition for TTX binding; thus, one would expect an increase in K_D of three–fivefold. If one now assumes by going to the Na-free solution a 50–70 mV change in membrane potential, which should decrease the K_D three–fivefold, then one would see no significant change in K_D. This was their observation.

KRUEGER: It is interesting that our results, which demonstrate voltage-dependent TTX and STX block, were obtained with BTX-activated sodium channels that were open in the steady state. This is in contrast to other work in which the membrane potential was held for long times at either hyperpolarized or depolarized potentials at which the channels were either closed or inactivated. It is of interest that Colatsky and Gadsby (1980. *J. Physiol.* (*Lond.*). 306:20P) reported using a heart preparation to study the steady-state block of open Na^+ channels under conditions where the channels were open but not inactivated. They found a small but significant shift in the steady-state binding constant for TTX in the same direction as we report here.

ADELMAN: Once TTX or STX block has been established, can you relieve it with a change in voltage and is it time dependent?

FRENCH: This has not been done quantitatively, but on changing the membrane potential one can see the relaxation to a new level of block within seconds.

E. CALCIUM CHANNELS FROM RAT BRAIN IN PLANAR BILAYERS.

Summary. Voltage-dependent calcium channels from rat brain were incorporated into planar bilayers using similar procedures to those described for sodium channels (29). A membrane fraction from rat brain median eminence was used as a source of these channels because that region consists primarily of nerve terminals involved in the release of neurohormones (30). Calcium channels are thought to be concentrated in nerve terminals where they are involved in the stimulus-coupled release of transmitter from synaptic vesicles, i.e., exocytosis (31). In the presence of symmetrical 0.25 M divalent cation, unitary fluctuations were observed with single channel conductances of 5 pS, 8.5 pS, and 5 pS for Ca^{++}, Ba^{++}, and Sr^{++}, respectively. Monovalent cations and anions were not measurably permeant. Membrane depolarization increased the mean open channel lifetimes (decreased the probability of closing) and decreased the mean closed lifetimes (increased the probability of opening). The open lifetimes were shorter in Ba^{++} that in Ca^{++} or Sr^{++}, suggesting a functional relationship between ion permeation (unit conductance) and voltage-gating (probability of closing).

The ability to study single channels, which have a much smaller unit conductance than sodium channels (5 pS vs 30 pS) was due to the development of a new current to voltage converter by Dr. French. Some of the sodium and calcium channel current fluctuations are still too fast to measure accurately and faster amplifiers are now being designed and fabricated.

The channels incorporated in the planar bilayer have been identified as calcium channels by several criteria detailed in ref. 29 a copy of which which follows. To the extent that a direct comparison can be made, these calcium channels have many properties in common with calcium channels in other tissues (32-34) including some that have been studied at the single channel level. (33,34). We believe that these channels may function to admit calcium ions into secretory nerve endings in the rat brain to act as a trigger for release of neurohormones. If so, these would be the first such calcium channels from mammalian central nervous system to be studied at the single channel level, and the first calcium channels of any kind to be reconstituted into artificial membranes. We may be in a position to answer a long-standing question as to whether ions can pass in both directions through calcium channels. While the normal direction is inward, Lee and Tsien (35) have shown that potassium and cesium can move outward through cardiac calcium channels (calcium could not be tested). It appears that in the experiments reported here, divalent ions are moving outward (inward movement has not been tested so far for technical reasons). Experiments are being designed to demonstrate divalent movement in both directions in the reconstituted system, in order to directly resolve this question.

We have proposed in the renewal application for this contract to incorporate calcium channels from heart into planar bilayers and to compare their properties with those of brain channels. This is of interest because of the pharmacological information available about cardiac calcium channels, particularly with respect to "organic calcium antagonists" (33,34). These substances, which are used to treat certain cardiac arrhythmias, block calcium entry through heart calcium channels. It will be of interest to test their effects on calcium channels from nerve.

Reprinted from Nature, Vol. 308, No. 5954, pp. 77-80, 1 March 1984

Voltage-dependent calcium channels from brain incorporated into planar lipid bilayers

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Many important physiological processes, including neurotransmitter release and muscle contraction[1-3], are regulated by the concentration of Ca^{2+} ions in the cell. Levels of cytoplasmic Ca^{2+} can be elevated by the entry of Ca^{2+} ions through voltage-dependent channels which are selective for Ca^{2+}, Ba^{2+} and Sr^{2+} ions[4-14]. We have measured currents through single, voltage-dependent calcium channels from rat brain that have been incorporated into planar lipid bilayers. Channel gating was voltage-dependent: membrane depolarization increased the channel open times and decreased the closed times. The channels were selective for divalent cations over monovalent ions. The well-known calcium channel blockers, lanthanum and cadmium, produced a concentration-dependent reduction of the apparent single-channel conductance. Contrary to expectations[14], the nature of the divalent cation carrying current through the channel affected not only the single-channel conductance, but also the channel open times, with mean open times being shortest for barium.

The median eminence was chosen as a potential source of Ca^{2+} channels because it is rich in nerve terminals which secrete releasing and inhibiting hormones that travel to the pituitary by means of the hypophysial portal system[15]. Channels were incorporated into bilayers from membrane vesicles that were made from rat brain median eminences using a method similar to that used for synaptosomes[16] or membrane vesicles[17] from whole brain. Planar bilayers containing phosphatidylethanolamine (33 mg ml^{-1}, bovine brain; Avanti Polar Lipids) and phosphatidylserine (26 mg ml^{-1}, bovine brain; Avanti) in decane were painted across a 100–250 μm hole in a polystyrene or Lexan partition separating two chambers[16,17], both (except where noted) containing (in mM) one of 250 $CaCl_2$ or $SrCl_2$ or $BaCl_2$ with 7.5 HEPES, 0.11 $CaCl_2$, 0.075 $MgCl_2$, 0.038 EGTA, pH 7.0 (that is, the divalent cation equilibrium potential was normally 0 mV). The *cis* side of the bilayer was defined as that side of the bilayer exposed to the membrane vesicles; the opposite (*trans*) side was held at virtual ground. Because, as inferred by their voltage dependence, the channels incorporated in the bilayer oriented with the extracellular ends of the channels facing the *trans* side and their cytoplasmic ends facing the *cis* side, voltages reported here correspond to the usual cellular convention (inside minus outside).

Within minutes following the addition of the membrane preparation to the *cis* side in the presence of 250 mM $CaCl_2$, $BaCl_2$ or $SrCl_2$, stepwise current fluctuations of 0.5 pA (Ca^{2+}, Sr^{2+}) or 0.85 pA (Ba^{2+}) were observed at +100 mV (Fig. 1*a–c*). The bottom current records in Fig. 1*a–c* show that fluctuations in current were not seen for any of the divalent cations at −100 mV. The current leak through the bilayer in the absence of any channel passing current was symmetrical at ±100 mV and corresponded to a 'naked bilayer' conductance of about 7.5 pS. Figure 1*d* shows superimposed single channel current steps for Ca^{2+}, Ba^{2+} and Sr^{2+} at +100 mV. Strontium and barium have been found to carry current through all Ca^{2+} channels studied, so that the ability of Sr^{2+} or Ba^{2+} to substitute for Ca^{2+} in maintaining Ca^{2+} currents is an important criterion used in identifying Ca^{2+} channels[3]. Figure 1 shows first that with equimolar concentrations of the three divalent cations the efficacy in carrying current at +100 mV was Ba > Ca = Sr. This agrees with reports for Ca^{2+} channels in other cell types[1-11]. Second, clear, well-defined current steps were seen for all three divalent cations at +100 mV, while current fluctuations were

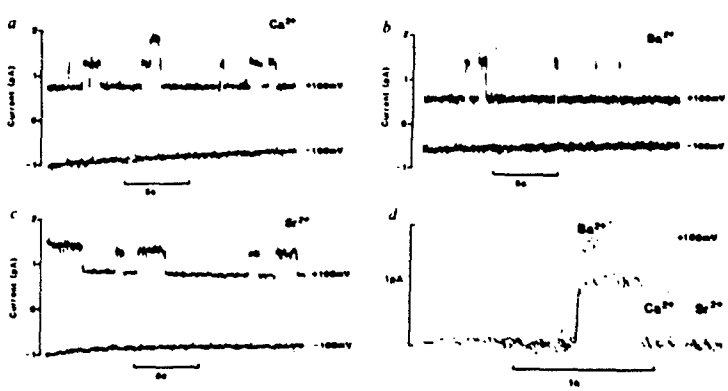

Fig. 1 *a*, Single-channel current fluctuations with Ca^{2+}. At +100 mV (top record), the current fluctuations were 0.5 pA in symmetric divalent cation solutions. In this experiment there were at least two channels in the bilayer. The holding potential was then changed to −100 mV (bottom record). At −100 mV, current fluctuations were not observed. The difference in current between −100 mV and +100 mV with all channels closed represents the bare bilayer conductance, about 7.5 pS. *b*, Single-channel current fluctuations with Ba^{2+}. At +100 mV, current fluctuations of about 0.85 pA with symmetric divalent cations were measured. At −100 mV, current fluctuations were not seen. *c*, Single-channel current fluctuations with Sr^{2+}. At +100 mV, current fluctuations of about 0.5 pA with symmetric divalent cations were measured. At −100 mV, current fluctuations were not observed. Hole diameter: 100 μm. *d*, Comparison of single-channel currents with Ba^{2+}, Ca^{2+} and Sr^{2+} at +100 mV. Current records taken in the presence of each ion were superimposed. In all cases, symmetric 250 mM divalent cations were present. **Methods:** Single-channel current fluctuations. The membranes were prepared from 10–15 rat brain median eminences, as described in ref. 16 with the omission of the sucrose gradient step. The membranes were suspended in 0.4 M sucrose and could be stored at −75 °C for at least 2 months without affecting the results. Identical results were obtained with both fresh and frozen preparations. The thawed synaptosomes in 0.4 M sucrose were then sonicated (probe type, Braun model 1510) for 20 s immediately before use. Addition of this membrane vesicle suspension (final concentration 0.1–0.5 μg protein ml^{-1}) resulted in stepwise current fluctuations[16,17]. All experiments were conducted at room temperature (19–25 °C). The side to which membrane vesicles were added was designated the *cis* side; the opposite (*trans*) side was held at virtual ground. Positive currents shown in the figures reflect positive charges moving outward, that is, from the *cis* side to the *trans* side. In all figures shown in this report except Fig. 2*c*, the divalent cation equilibrium potential was 0 mV. The cut-off frequency of the current to voltage converter was about 600 Hz. All experiments were recorded on FM tape at 600 Hz and, unless otherise noted, records were filtered at 100 Hz on playback. Unless stated otherwise, the diameter of the holes onto which the bilayers were painted was 250 μm. The slow change in current seen in Figs 1*a* and *c* at −100 mV reflected the residual relaxation of the capacitative current following the voltage step from +100 mV to −100 mV which preceded the record shown.

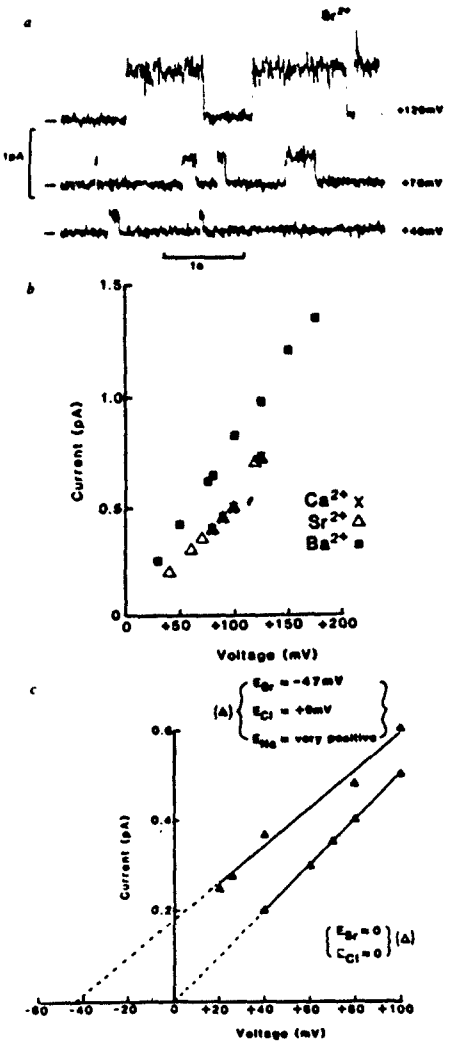

Fig. 2 Current voltage relations for single calcium channels. *a*, Effects of voltage on the magnitude of single-channel currents. Records were taken on the same membrane with symmetric 250 mM $SrCl_2$. The solid horizontal bars in each record indicate the current level with all channels closed. Hole diameter: 100 μm. Above +100 mV, there is a pronounced increase in current fluctuations in the open state of the channel. *b*, Current–voltage relationship for single calcium channels with Ca^{2+}, Ba^{2+} and Sr^{2+}. Symmetric 250 mM divalent cations, that is, the zero-current potential and divalent cation equilibrium potential were at 0 mV. Single-channel conductances for Ca^{2+}, Ba^{2+} and Sr^{2+} were 5 pS, 8.5 pS and 5 pS, respectively. *c*, Single-channel current voltage relations with symmetrical 250 mM $SrCl_2$ (Δ, same data as in *b*) and with 357 mM NaCl, 6.1 mM $SrCl_2$ on the *trans* side and 250 mM $SrCl_2$ on the *cis* side (▲). The equilibrium potentials (calculated from concentrations) for these experiments were: Δ (symmetric), E_{Sr} = E_{Cl} = 0 mV; ▲ (asymmetric), E_{Sr} = −47 mV, E_{Cl} = +9 mV, E_{Na} = nominally plus infinity. The slopes of the linear regression (solid)

not seen at −100 mV. Thus, the gating of these single channels is voltage-dependent. Since single-channel current fluctuations continued to be observed for many seconds following a voltage step, the channels under these experimental conditions do not completely inactivate (compare refs 9–11). Thus far, with the conditions described in this paper, we have been able to incorporate Ca^{2+} channels on virtually every attempt (>50) and channels can frequently be studied for periods of 30 to 60 min following incorporation.

Single-channel current–voltage relations for these cations are shown in Fig. 2. Figure 2*a* shows representative single channel records at three potentials with Sr^{2+} as the current carrier. Figure 2*b* shows that the single-channel current with either Ca^{2+}, Ba^{2+} or Sr^{2+} as the current carrier was proportional to applied voltage over a range of about 145 mV. The single-channel conductances with Ca^{2+}, Ba^{2+} and Sr^{2+} as the current carriers were 5 pS, 8.5 pS and 5 pS, respectively, indicating that with symmetric 250 mM divalent cations, Ba^{2+} moves through the channel about 1.7 times faster than Ca^{2+} or Sr^{2+}. At potentials above +100 mV, open channel current noise with all three divalent cations increased substantially (see Fig. 2*a*). Reducing the Ca^{2+} concentration from 250 to 150 mM had no effect on the single-channel conductance, suggesting that at the levels of cations used in these experiments, the Ca^{2+} channels were saturated with respect to the current carrier[3,9,11]. The use of symmetric solutions eliminates surface potential differences, and channel saturation eliminates variation in the degree of occupancy by the permeant ions. Thus, the different single-channel conductances should reflect differences among the limiting rates of transport of these ions through the channels. Negative to +30 mV, the single-channel currents were too small and too brief to measure. Patch clamp studies on intact cultured rat heart muscle and clonal rat pituitary cells suggest that single-channel Ba^{2+} currents increase linearly over a 50–60 mV range[4,5] and that Ba^{2+} carries more current than Ca^{2+} through single channels in *Helix* neurones[6].

Direct evidence of selectivity for divalent cations over monovalent cations is shown in Fig. 2c. In this experiment, the *trans* $SrCl_2$ solution was replaced with an isotonic solution of NaCl. Although the channels were open long enough to be measured only at potentials more depolarized than +20 mV, the single channel current–voltage relationship was linear and could be extrapolated to a potential (−44 mV) that was very close to the divalent cation equilibrium potential (E_{Sr}, −47 mV). By contrast, the chloride equilibrium potential (E_{Cl}) was +9 mV and the sodium equilibrium potential (E_{Na}) was nominally plus infinity. This result rules out the possibility that these channels select for Na^+ or Cl^-. In other experiments, a high concentration (200 mM) of KCl was added to the *cis* side with 150–250 mM $CaCl_2$ or $BaCl_2$ present on both sides. In these experiments (results not shown), there was no change in the single channel current at any potential. These results also support the conclusion that these channels are selective for divalent cations over monovalent ions.

As was illustrated in Fig. 1*a–c*, the channels are more likely to be open at positive potentials. We have also analysed the effect of changing potential on the single-channel open and closed times, which reflect the probabilities of closing and opening. Using either Ca^{2+}, Ba^{2+} or Sr^{2+} as the current carrier, decreasing the membrane potential from +100 mV to +50 mV caused the channel open times to decrease, and the closed times (intervals between openings) to increase (data shown for Ba^{2+}, Fig. 3). At +100 mV, the mean open time of the channel with Ba^{2+} as the current carrier was about 127 ms (Fig. 3*b*). Reducing the potential to +75 mV and then to +50 mV decreased the mean open time of the channel to 76 ms and to 29 ms. If the mean open time continues to decrease as the membrane potential is reduced further, then the channel open times should have been in the millisecond range around 0 mV, a value similar to those obtained in patch clamp experiments on other preparations[4–6]. Reducing the potential not only decreased channel

3c). From +100 mV to +50 mV, the closing rate increased 4.4-fold and the opening rate decreased 2.5-fold indicating that the probability of a channel being open changed approximately 10-fold for this 50 mV change in membrane potential, a value similar to that found for Ba^{2+}-conducting channels in cultured heart muscle cells[4].

Channel open times were affected by the species of ion carrying the current through the channel as well as by voltage (contrast ref. 22). Throughout the range of potentials studied, channels with Ba^{2+} carrying the current had a shorter mean open time than with Ca^{2+} or Sr^{2+} as current carriers (Fig. 3*d*). At +100 mV, the single-channel mean open times with Ba^{2+}, Sr^{2+} and Ca^{2+} were 127 ms, 385 ms and 454 ms, respectively. Thus, there is a parallel between the sequence of mean open times, and the sequence of mean transit times for the permeant ions (0.4 μs for Ba^{2+}; 0.7 μs for Ca^{2+} and Sr^{2+}, calculated from the unit current at +100 mV). A similar relationship can be derived from open times and unit conductances observed when different alkali cations carry current through the acetylcholine receptor channel[18,19]. In addition, the order of the mean open times ($Ba^{2+} < Sr^{2+} < Ca^{2+}$) is the same as the order of apparent affinities of these ions for Ca^{2+} channels in rat brain synaptosomes[9]. These results are consistent with the general idea that occupancy of a channel by a permeant ion can impede channel closing, a conclusion also reached in studies of squid axon potassium channels[20].

Calcium entry into a wide variety of cells is mediated by voltage-activated channels that are permeable to the cations Ca^{2+}, Ba^{2+} and Sr^{2+} (see Fig. 1; for review see ref. 3). The exact mechanism of ion permeation is unknown, but one important step may be the association of Ca^{2+} with a binding site in the channel. There is good evidence that in many types of cells, lanthanum and a variety of transition metals block Ca^{2+} movement through the channels by competing with Ca^{2+} for this site. To date, there are no data at the single channel level on the mechanism of block by inorganic ions. We found that lanthanum reduces the single channel current in a dose-dependent manner (Fig. 4). In the presence of 250 mM Sr^{2+}, 145 μM La^{3+} reduced the single channel current by 50%. In addition to lanthanum, cadmium and manganese also reduced the single channel currents, with the order of potency being: $La^{3+} > Cd^{2+} \gg Mn^{2+}$.

The selectivity among Ca^{2+}, Ba^{2+} and Sr^{2+}, and for divalent over monovalent cations, the small single-channel conductances, the voltage dependence, and the block by lanthanum and cadmium, reported here, are considered to be properties common to all Ca^{2+} channels[1-3]. Based on these criteria, these channels closely resemble voltage-dependent calcium channels that have been studied in a variety of excitable tissues. Of several unresolved questions about Ca^{2+} channels, the following appear to be especially well-suited for investigation using the bilayer system:

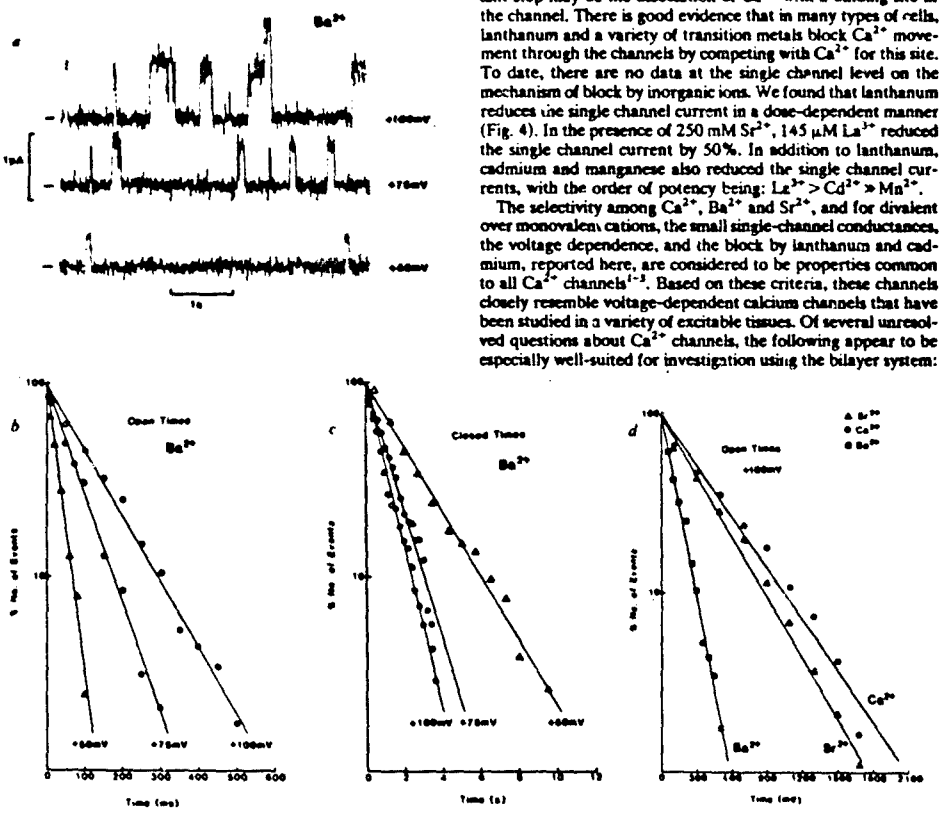

Fig. 3 Voltage-dependence of single-channel open times and closed times. *a*, Original record of single-channel current fluctuations with Ba^{2+} as the current carrier at +50 mV, +75 mV and +100 mV. The solid bars show the level at which all the channels were closed. *b*, Effect of voltage on the distribution of single-channel open times. Open-time distributions were determined from the same experiment shown in 3*a*. The open times were determined from single-level fluctuations that terminated in a closure; multiple-level fluctuations were rare and were not counted. The distributions of open times at +50 mV, +75 mV, +100 mV could be described in each case by a single exponential[1]. Plotted on a logarithmic scale on the ordinate is the percentage of events that lasted at least as long as the time indicated on the abscissa. The total number of events, the slopes and correlation coefficients of the linear regression lines through the log-transformed data were at +50 mV, 142, $-34.7\,s^{-1}$, −0.993; at +75 mV, 77, $-13.1\,s^{-1}$, −0.998; and at +100 mV, 75, $-7.9\,s^{-1}$, −0.999. *c*, Effect of voltage on the distribution of times between openings from the same experiment. The distributions of closed times at +50 mV, +75 mV and +100 mV could also be described by single exponentials. The total number of events, the slopes and correlation coefficients of the linear regression lines through the log-transformed data were at +50 mV, 142, $-0.38\,s^{-1}$, −0.993; at +75 mV, 77, $-0.76\,s^{-1}$, −0.985; and at +100 mV, 71, $-0.94\,s^{-1}$, −0.997. *d*, Effects of Ba^{2+}, Ca^{2+} and Sr^{2+} on the distribution of single-channel open times at +100 mV. The total number of events and the slopes and correlation coefficients of the linear regression lines through the log-transformed data were with Ba^{2+}, 75, $-7.9\,s^{-1}$, −0.999; with Sr^{2+}, 124, $-2.6\,s^{-1}$, −0.996; and with Ca^{2+}, 82, $-2.2\,s^{-1}$, −0.985.

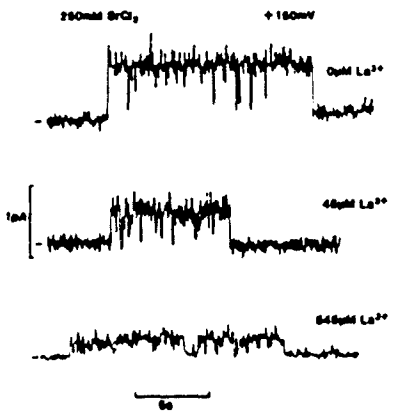

Fig. 4 Block of single-channel strontium currents by lanthanum. Representative single channel current steps in the presence of 250 mM $SrCl_2$ in 0, 45 and 545 μM $LaCl_3$ on the *cis* side. The top and middle records were filtered at 100 Hz and the bottom record was filtered at 20 Hz on playback.

(1) Can permeant divalent cations move through the channels in both directions? In the presence of physiological Ca^{2+} gradients, current through Ca^{2+} channels is inward. Our results probably reflect an outward movement *of* divalent cations through the open channels. Bidirectional movement could be verified in the presence of the appropriate divalent cation concentration gradients to allow measurable currents at potentials where the channels are open. (2) Are Ca^{2+} channels modulated directly by neurohormones (for example, catecholamines) or via a cyclic AMP–protein phosphorylation cascade[1,2]? This hypothesis could be tested in the planar bilayer system by adding either the neurohormones or the constituents necessary for protein phosphorylation. (3) How do clinically important 'Ca-antagonists' affect single Ca^{2+} channels[21]? The side of the channels at which these agents act and the reversibility of their action could easily be investigated. Finally, the planar bilayer system may also provide the opportunity to compare Ca^{2+} channels from different tissues, such as heart and smooth muscle, under well-defined conditions.

We thank W. J. Lederer and D. A. Nachshen for helpful discussions, M. P. Blaustein, M. Cannell, E. Marban and J. F. Worley III for comments on the manuscript, E. Bamberg and P. Läuger for insights into the planar bilayer system and ion channel function, K. Gregerson and M. Selmanoff for introducing us to the median eminence preparation and D. Darragh for preparing the figures. This work was supported by a Grant-in-aid from the American Heart Association, Maryland Affiliate (M.T.N.), and the Alexander von Humboldt-Stiftung (M.T.N.), the Bressler Research Fund of the University of Maryland (R.J.F. and B.K.K.) and contract DAMD17-82-C-2188 from the US Army Medical Research and Development Command (B.K.K. & R.J.F.).

Received 21 July; accepted 29 November 1983.

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